CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

III. Periodic and Progressive Variations in Normal Rabbits.

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The results of a large series of determinations of calcium and inorganic phosphorus in the blood of normal rabbits were analyzed in previous papers (1, 2) from the standpoint of grouped material with a view to establishng certain basic facts concerning the amounts of these substances in the blood, the relations that obtain, and the extent of the variations that occur under given experimental conditions. The results have been analyzed also with respect to the values obtained for consecutive examinations of a given group of animals with especial reference to the occurrence of periodic and progressive variations in the calcium and inorganic phosphorus content of the blood. This aspect of the investigation will be presented in the present paper.

Material and Methods.

The data used in this paper are derived from experiments which were described in detail in the two preceding papers (1, 2). For present purposes, it is sufficient to say that we have used data from 5 groups of normal, male rabbits differing somewhat as to age, breed, the time covered by the observations, and the frequency with which blood examinations were made. The pertinent features of the experiments may be summarized as follows:

Group	No. of animals	No. of examinations	Observation period
I	10	216	Oct. 8, 1926, to May 18, 1927
II	5	85	Dec. 1, 1926, to June 8, 1927
III	10	143	Jan. 14, to July 1, 1927
IV	11	153	Mar. 11, to July 1, 1927
V	111	111	Oct. 8, 1926, to June 8, 1927

Between October 8, 1926, and July 1, 1927, determinations of calcium and inorganic phosphorus were made on 708 samples of blood from 147 rabbits. Group V was composed of 8 subgroups (2), the animals of which were examined only once; the other animals were examined repeatedly.

RESULTS.

The results are presented in the form of tabulated summaries and a series of text-figures. Tables I to VI give the actual values obtained for successive determinations of calcium and inorganic phosphorus as well as the relations between the two substances. The curves in Text-figs. 1 to 9 represent values that have been smoothed by the formula $\frac{A + 2B + C}{4}$.

TABLE I.

Group I. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 8	13.74 ± .198	0.93	6.79	5.18 ± .189	0.89	17.18
" 15	13.22 ± .215	1.01	7.65	4.55 ± .098	0.46	10.11
" 21	15.39 ± .247	1.16	7.53	4.23 ± .168	0.79	18.68
" 29	15.15 ± .215	1.01	6.64	4.15 ± .087	0.41	9.88
Nov. 5	16.07 ± .363	1.70	10.56	4.77 ± .141	0.66	13.84
" 12	13.94 ± .26	1.22	8.78	5.02 ± .115	0.54	10.76
" 19	15.16 ± .133	0.62	4.08	4.98 ± .109	0.51	10.24
" 26	14.64 ± .211	0.99	6.78	4.70 ± .115	0.54	11.49
Dec. 3	15.19 ± .294	1.38	9.08	4.52 ± .094	0.44	9.73
" 10	15.48 ± .136	0.64	4.13	4.26 ± .094	0.44	10.33
" 17	15.48 ± .198	0.93	6.00	4.10 ± .147	0.69	16.83
" 31	15.90 ± .094	0.44	2.77	4.56 ± .070	0.33	7.24
Jan. 14	15.4 ± .100	0.47	3.05	4.35 ± .113	0.53	12.18
" 28	16.9 ± .209	0.98	5.81	3.94 ± .060	0.28	7.11
Feb. 11	15.3 ± .149	0.70	4.58	4.14 ± .062	0.29	7.00
" 25	15.6 ± .122	0.57	3.65	4.15 ± .092	0.43	10.36
Mar. 11	15.8 ± .108	0.51	3.20	3.89 ± .049	0.23	5.91
" 25	15.6 ± .109	0.51	3.27	3.84 ± .045	0.21	5.47
Apr. 8	15.3 ± .173	0.81	5.29	3.75 ± .100	0.47	12.53
" 22	15.7 ± .207	0.92	5.86	3.43 ± .065	0.29	8.45
May 4	15.5 ± .119	0.53	3.42	3.25 ± .063	0.28	8.62
" 18	17.6 ± .119	0.53	3.01	3.95 ± .047	0.21	5.32

TABLE II.

Group II. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Dec. 1	13.9 ± .368	0.82	8.79	3.83 ± .084	0.28	7.23
Jan. 5	15.3 ± .244	0.81	5.29	5.03 ± .133	0.44	8.75
" 19	15.6 ± .184	0.61	3.92	4.66 ± .056	0.19	3.99
Feb. 4	17.2 ± .235	0.78	4.53	4.38 ± .118	0.39	8.90
" 11*	16.6 ± .965	3.20	19.28	4.41 ± .103	0.34	7.71
" 16	15.4 ± .201	0.67	4.32	4.32 ± .133	0.44	10.19
" 28	15.6 ± .253	0.84	5.38	3.85 ± .045	0.15	3.89
Mar. 2	15.0 ± .214	0.71	4.73	4.07 ± .021	0.07	1.74
" 9	14.7 ± .362	1.20	8.16	4.10 ± .130	0.43	10.49
" 16	14.8 ± .193	0.64	4.32	3.64 ± .078	0.26	7.14
" 23	14.8 ± .089	0.30	2.00	3.72 ± .066	0.22	5.91
Apr. 6	15.5 ± .187	0.62	4.06	4.53 ± .181	0.60	13.25
May 4	15.4 ± .259	0.86	5.58	3.87 ± .118	0.39	10.08
" 6	15.2 ± .193	0.64	4.21	4.21 ± .072	0.24	5.70
" 11	17.0 ± .274	0.91	5.35	3.90 ± .075	0.25	6.41
" 25	16.6 ± .145	0.48	2.89	3.76 ± .094	0.31	8.24
June 8	15.8 ± .259	0.86	5.44	3.61 ± .115	0.38	10.53

* This group contained one value 22.8 and another 13.8.

TABLE III.

Group III. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 14	14.8 ± .181	0.89	6.01	5.75 ± .104	0.51	8.87
" 26	16.3 ± .256	1.26	7.73	5.24 ± .112	0.55	10.50
Feb. 9	15.0 ± .122	0.57	3.80	5.33 ± .113	0.53	9.94
" 18	15.3 ± .151	0.71	4.64	5.13 ± .087	0.41	7.99
Mar. 4	15.2 ± .159	0.78	5.13	4.74 ± .083	0.41	8.65
" 18	15.3 ± .119	0.56	3.66	4.85 ± .088	0.39	8.04
" 30	14.8 ± .119	0.56	3.78	5.11 ± .094	0.44	8.61
Apr. 13	14.8 ± .109	0.51	3.45	4.44 ± .092	0.43	9.68
" 27	14.9 ± .109	0.51	3.42	4.34 ± .079	0.37	8.53
May 11	16.4 ± .143	0.67	4.09	4.17 ± .115	0.54	12.95
" 25	16.3 ± .128	0.60	3.68	3.93 ± .111	0.52	13.23
June 8	16.6 ± .119	0.56	3.37	3.70 ± .094	0.44	11.89
" 17	17.1 ± .267	1.25	7.31	4.29 ± .092	0.43	10.02
July 1	17.4 ± .122	0.57	3.28	4.28 ± .107	0.50	11.68
Sept. 15	14.9 ± .186	0.87	5.84	4.24 ± .100	0.47	11.08
Oct. 19	15.9 ± .256	1.20	7.55	3.99 ± .109	0.51	12.78

TABLE IV.

Group IV. Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Mar. 11	15.2 ± .195	0.96	6.32	5.90 ± .126	0.62	10.51
" 18	15.1 ± .110	0.54	3.58	5.55 ± .112	0.55	9.91
" 25	14.9 ± .087	0.43	2.89	6.23 ± .094	0.46	7.38
Apr. 1	15.2 ± .115	0.57	3.72	5.84 ± .126	0.62	10.62
" 8	15.5 ± .110	0.54	3.48	5.01 ± .100	0.49	9.78
" 15	15.3 ± .122	0.60	3.92	4.48 ± .087	0.43	9.60
" 22	15.0 ± .117	0.58	3.83	4.93 ± .107	0.53	10.67
" 29	15.2 ± .230	1.13	7.43	4.71 ± .087	0.43	9.13
May 6	15.6 ± .159	0.78	5.00	4.61 ± .107	0.53	11.39
" 13	16.2 ± .106	0.52	3.21	4.51 ± .122	0.60	13.30
" 25	17.0 ± .146	0.72	4.24	4.58 ± .075	0.35	7.64
June 8	16.8 ± .120	0.59	3.51	4.10 ± .075	0.37	9.02
" 17	17.6 ± .191	0.94	5.34	4.57 ± .069	0.34	7.66
July 1	17.2 ± .068	0.34	1.95	4.42 ± .106	0.52	11.77
Sept. 15	15.4 ± .195	0.96	6.23	4.39 ± .073	0.36	8.20
Oct. 25	15.2 ± .147	0.69	4.54	4.28 ± .075	0.35	10.51

TABLE V.

Group. V. Values for Consecutive Monthly Groups.

Date	Calcium			Inorganic phosphorus		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 8	13.7 ± .198	0.93	6.79	5.18 ± .189	0.89	17.18
Nov. 17	14.7 ± .066	0.31	2.11	5.22 ± .124	0.58	11.11
Jan. 7–14	15.3 ± .169	1.15	7.52	5.06 ± .135	0.92	18.18
Feb. 4	17.5 ± .097	0.48	2.74	5.33 ± .146	0.72	13.51
Mar. 11	15.0 ± .128	1.08	7.20	5.48 ± .108	0.91	16.61
Apr. 29	15.1 ± .155	0.73	4.83	5.53 ± .068	0.32	5.77
May 25	16.2 ± .200	0.89	5.49	5.81 ± .245	1.09	18.76
June 8	17.5 ± .230	0.97	5.53	5.92 ± .136	0.57	9.63

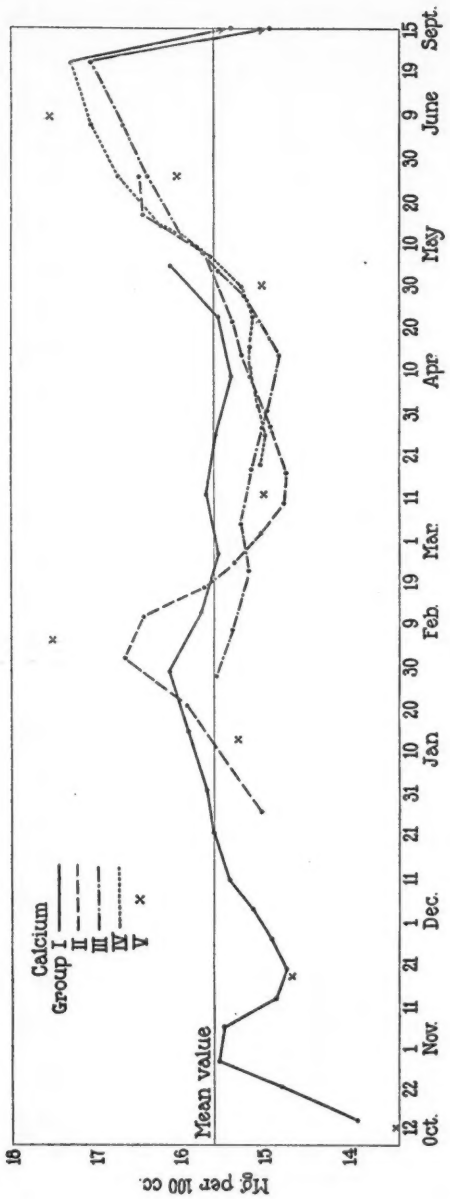
TABLE VI.

Group Means for Consecutive Determinations: Sum, Product, Calcium-Inorganic Phosphorus Ratio, Product-Sum Ratio, and Sum of Both Ratios.

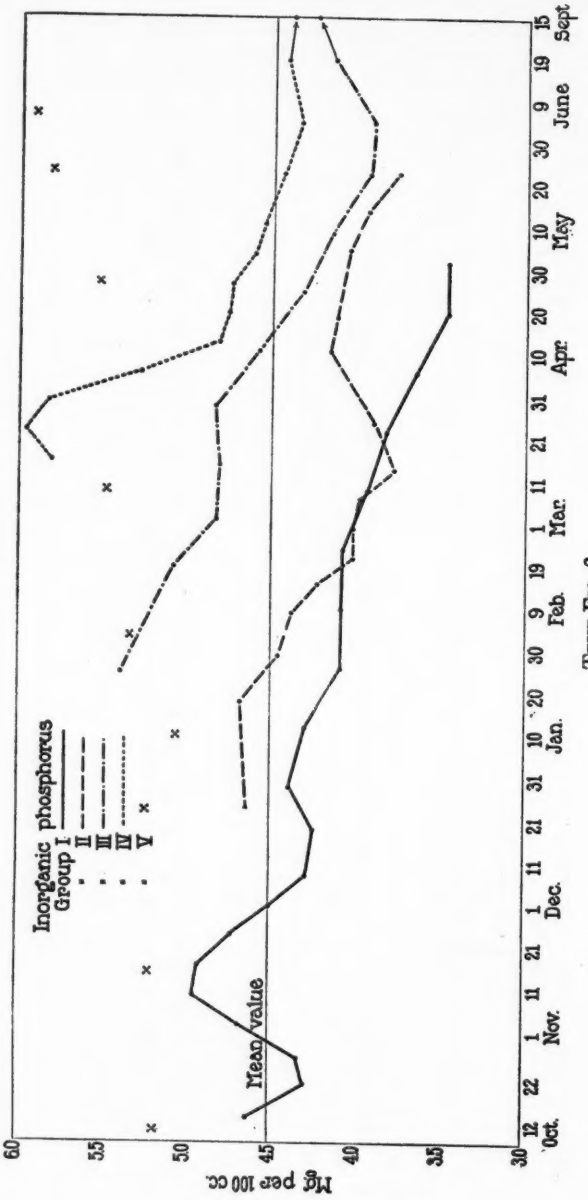
Group	Date	Sum	Product	Calcium-phosphorus ratio	Product-sum ratio	Sum of two ratios
I	Oct. 8	18.9	71.2	2.65	3.76	6.41
	" 15	17.8	60.2	2.91	3.39	6.30
	" 21	19.6	65.1	3.64	3.32	6.96
	" 29	19.3	62.9	3.65	3.26	6.91
	Nov. 5	21.2	78.3	3.44	3.70	7.14
	" 12	19.0	70.0	2.77	3.69	6.46
	" 19	20.1	75.5	3.04	3.75	6.79
	" 26	19.3	68.8	3.12	3.56	6.68
	Dec. 3	19.7	68.7	3.36	3.49	6.85
	" 10	19.7	65.9	3.63	3.34	6.97
	" 17	19.6	63.5	3.77	3.24	7.01
	" 31	20.5	72.5	3.48	3.54	7.02
	Jan. 14	19.8	67.1	3.54	3.39	6.93
	" 28	20.9	66.6	4.29	3.19	7.48
	Feb. 11	19.4	63.2	3.68	3.26	6.94
	" 25	19.7	64.7	3.75	3.28	7.03
	Mar. 11	19.7	61.3	4.01	3.12	7.13
	" 25	19.5	60.1	4.09	3.08	7.17
	Apr. 8	19.0	57.2	4.07	3.01	7.08
	" 22	19.1	53.9	4.58	2.82	7.40
	May 5	18.8	50.4	4.77	2.68	7.45
	" 18	21.8	70.3	4.51	3.22	7.73
II	Dec. 1	17.8	53.4	3.64	3.00	6.64
	Jan. 5	20.3	77.0	3.04	3.78	6.82
	" 19	20.2	72.5	3.34	3.58	6.92
	Feb. 4	21.6	75.5	3.93	3.49	7.42
	" 11	21.0	72.3	3.72	3.44	7.16
	" 16	19.7	66.4	3.55	3.37	6.92
	" 28	19.4	59.9	4.04	3.09	7.13
	Mar. 2	19.0	60.9	3.67	3.19	6.86
	" 9	18.8	60.2	3.58	3.20	6.78
	" 16	18.4	53.7	4.06	2.92	6.98
	" 23	18.5	54.9	3.96	2.97	6.93
	Apr. 6	20.0	70.0	3.41	3.50	6.91
	May 4	19.3	59.7	3.98	3.09	7.07
	" 6	19.4	64.1	3.62	3.29	6.91
	" 11	20.9	66.2	4.35	3.17	7.52
	" 25	20.4	62.4	4.41	3.06	7.47
	June 8	19.4	56.9	4.36	2.93	7.29

TABLE VI—*Concluded.*

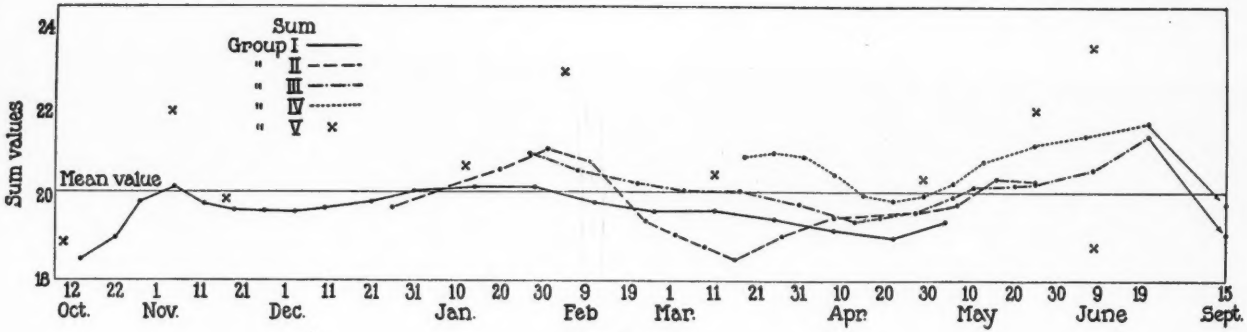
Group	Date	Sum	Product	Calcium-phosphorus ratio	Product-sum ratio	Sum of two ratios
III	Jan. 14	20.6	85.1	2.57	4.13	6.70
	" 26	21.5	85.4	3.11	3.97	7.08
	Feb. 9	20.3	80.0	2.81	3.94	6.75
	" 18	20.4	78.5	2.98	3.85	6.83
	Mar. 4	19.9	72.0	3.21	3.62	6.83
	" 18	20.2	74.2	3.15	3.67	6.82
	" 30	19.9	75.6	2.90	3.80	6.70
	Apr. 13	19.2	65.7	3.33	3.42	6.75
	" 27	19.2	64.7	3.43	3.37	6.80
	May 11	20.6	68.4	3.93	3.32	7.25
	" 25	20.2	64.1	4.15	3.17	7.32
	June 8	20.3	61.4	4.49	3.02	7.51
	" 17	21.4	73.4	3.99	3.43	7.42
	July 1	21.7	74.5	4.07	3.43	7.50
	Sept. 15	19.1	63.2	3.51	3.31	6.82
IV	Mar. 11	21.0	89.7	2.58	4.25	6.83
	" 18	20.7	83.5	2.71	4.05	6.76
	" 25	21.1	92.6	2.38	4.39	6.77
	Apr. 1	21.0	88.5	2.60	4.21	6.81
	" 8	20.5	77.7	3.09	3.79	6.88
	" 15	19.8	68.5	3.41	3.46	6.87
	" 22	19.9	74.0	3.05	3.71	6.76
	" 29	19.9	71.9	3.23	3.60	6.83
	May 6	20.2	71.8	3.38	3.55	6.93
	" 13	20.7	72.9	3.58	3.52	7.10
	" 25	21.6	77.9	3.71	3.61	7.32
	June 8	20.9	68.9	4.10	3.30	7.40
	" 17	22.2	80.4	3.85	3.62	7.47
	July 1	21.6	76.0	3.89	3.52	7.41
	Sept. 15	19.8	67.6	3.51	3.41	6.92
V	Oct. 8	18.9	71.5	2.72	3.74	6.46
	Nov. 17	19.9	76.4	2.84	3.84	6.68
	Jan. 12	20.3	76.9	3.14	3.76	6.90
	Feb. 4	22.9	93.5	3.35	4.07	7.42
	Mar. 11	20.5	82.0	2.82	3.98	6.80
	Apr. 29	20.4	83.0	2.84	4.06	6.89
	May 25	22.0	93.6	2.90	4.11	7.01
	June 8	23.5	104.0	2.98	4.41	7.39


Calcium
Group I
II
III
IV
V
Mean value
Mg. per 100 cc.
18
17
16
15
14
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan Feb. Mar Apr May June Sept.

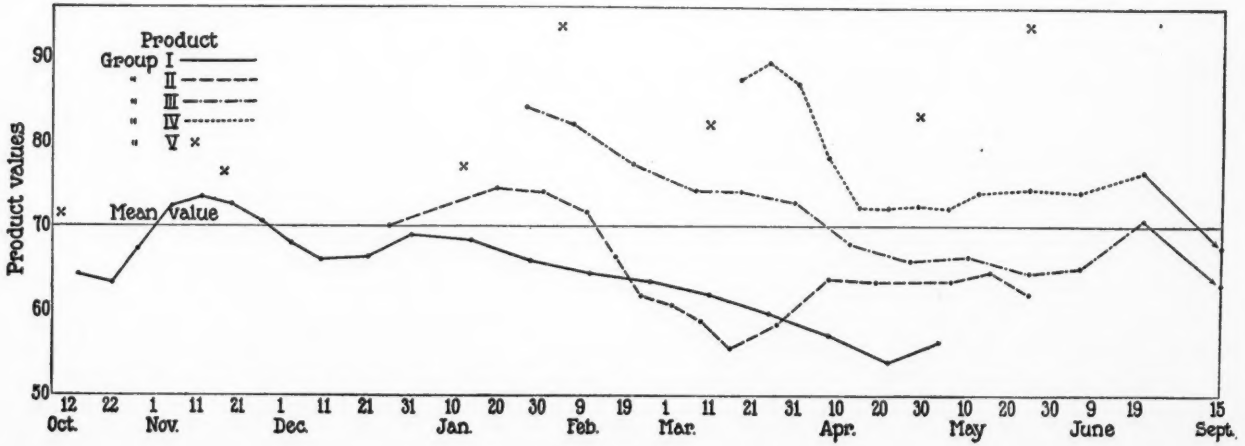
TEXT-FIG. 1.


Inorganic phosphorus
Group I
II
III
IV
V
Mean value
Mg per 100 cc.
60
55
50
45
40
35
30
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 1 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

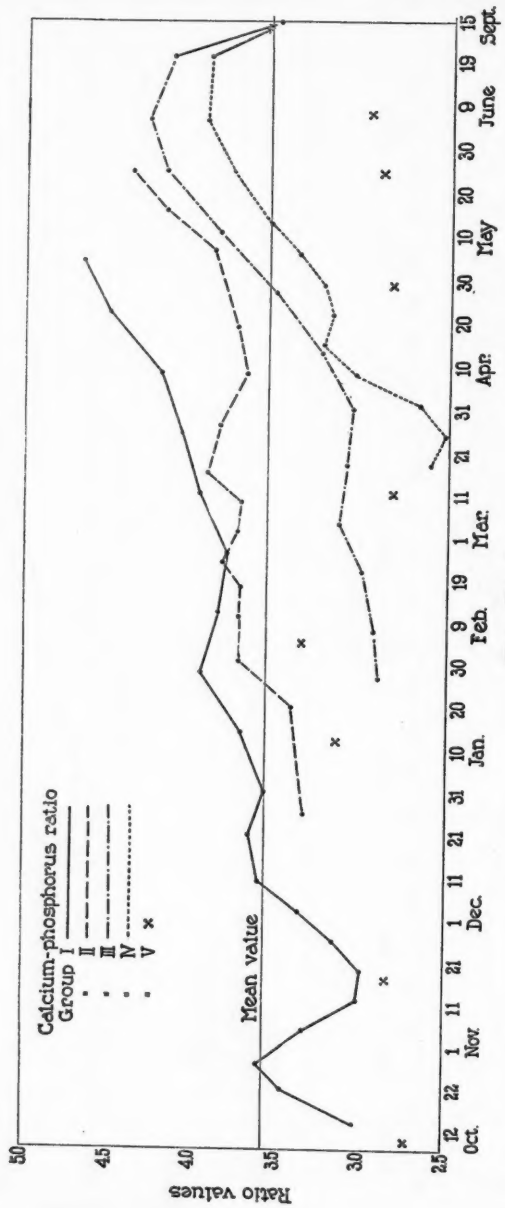
TEXT-FIG. 2.


Sum
Group I
" II
" III
" IV
" V
Mean value
Sum values
24
22
20
18
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb Mar. Apr May June Sept.

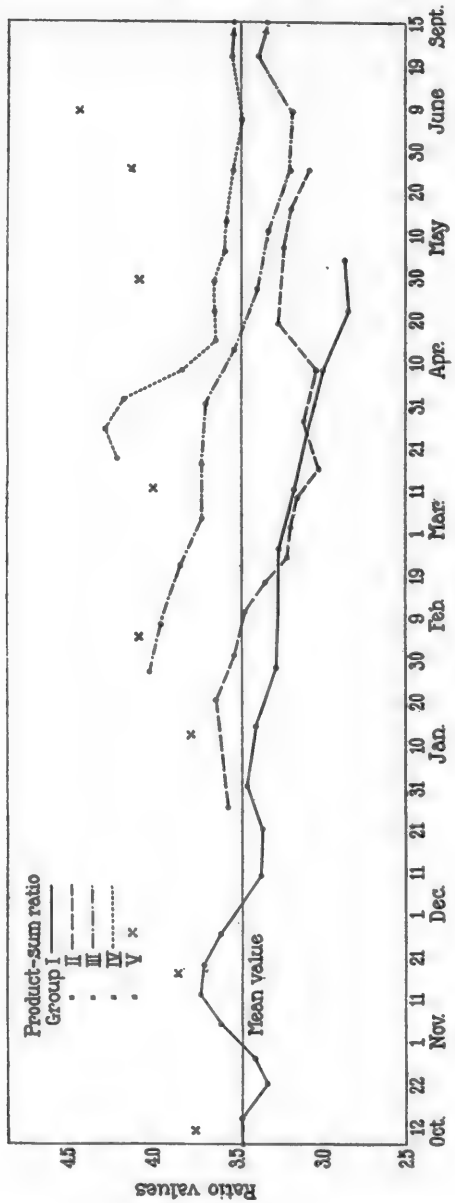
TEXT-FIG. 3.



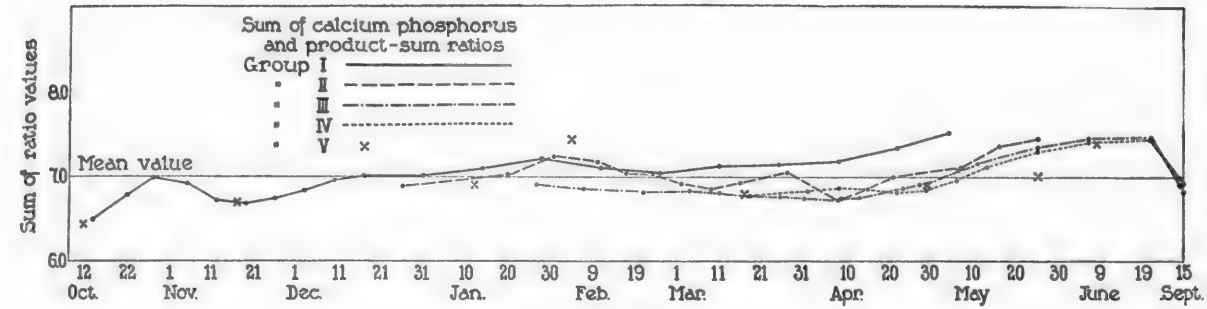
TEXT-FIG. 4.


Calcium-phosphorus ratio
Group I
II
III
IV
V
Mean value
Ratio values
50
45
40
35
30
25
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

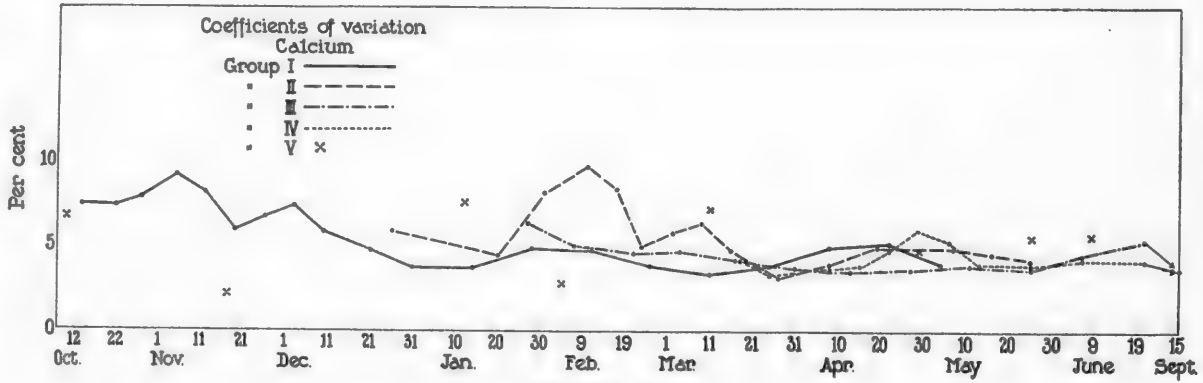
TEXT-FIG. 5.


Product-sum ratio
Group I
II
III
IV
V
Mean value
Ratio values
45
40
35
30
25
Oct. 12 22 Nov. 1 11 21 Dec. 1 11 21 31 Jan. 10 20 30 Feb 9 19 Mar 1 11 21 31 Apr. 10 20 30 May 10 20 30 June 9 19 Sept. 15

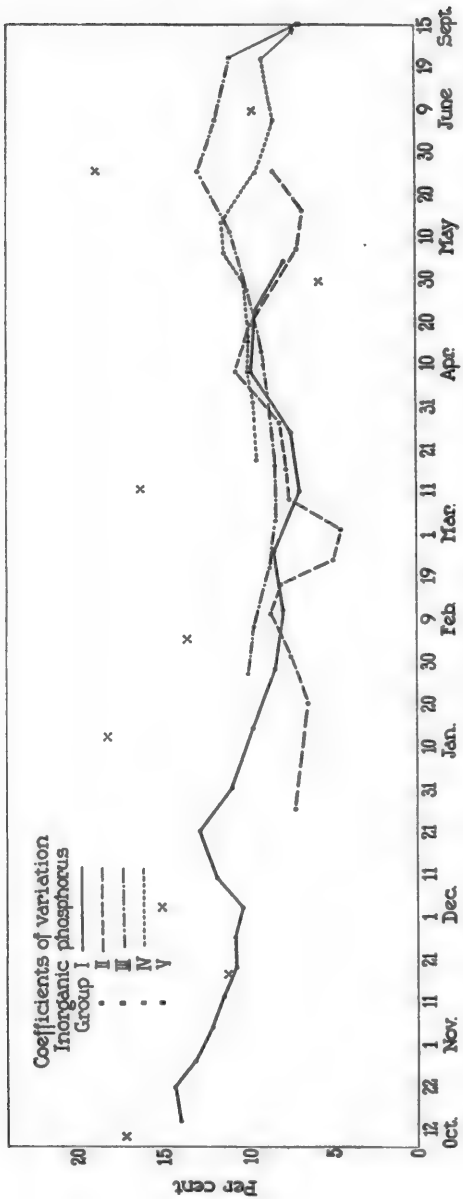
TEXT-FIG. 6.


Sum of calcium phosphorus
and product-sum ratios
Group I
II
III
IV
V
Sum of ratio values
8.0
7.0
6.0
Mean value
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

TEXT-FIG. 7.


Coefficients of variation
Calcium
Group I
II
III
IV
V
Per cent
10
5
0
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June Sept.

TEXT-FIG. 8.


Coefficients of variation
Inorganic phosphorus
Group I
II
III
IV
V
Per cent
0
5
10
15
20
12 22 1 11 21 1 11 21 31 10 20 30 9 19 1 11 21 31 10 20 30 10 20 30 9 19 15
Oct. Nov. Dec. Jan. Feb Mar. Apr. May June Sept.

TEXT-FIG. 9.

DISCUSSION AND CONCLUSIONS.

An examination of the figures given in Tables I to VI and the curves in Text-figs. 1 to 9 shows at once that both the absolute values for calcium and inorganic phosphorus and the relative amounts of these substances exhibited an interesting series of variations in all of the animals studied. The changes observed in different groups of animals were not identical, but there were certain general features that were common to all, as may be seen by comparing the curves in Text-figs. 1 to 9. The course of events is more difficult to follow from the tabulated results.

In practically all instances, there are variations of two types. One of these is progressive in character and represents a gradual change of values from a lower to a higher, or from a higher to a lower level. This gradual change of level is accompanied in some instances by a clearly defined series of periodic variations, the positive and negative phases of which extend over periods of several weeks or months. In other cases, clearly defined periodic increases are absent, but the gradual change in level is marked by periods of stabilization at a given level, or by a temporary increase or decrease in the rate of change. The tabulated results show, however, that neither of these conditions represents a continuous movement in a given direction, but that the periodic variation is itself produced by a succession of pendulum-like fluctuations. Moreover, it will be seen that, to some extent, the progressive increase or decrease in values is in reality a long period variation as the high or low levels are not maintained indefinitely. This is particularly true of calcium as may be seen by reference to the tables and text-figures for Groups III and IV (Tables III and IV and Text-fig. 1).

From October until July, the general trend of calcium values is slightly upward (Tables I to V and Text-fig. 1). The outstanding feature of the condition shown by blood calcium is, however, the occurrence of a series of periodic increases and decreases. From October to December, 1926, consecutive observations were made on only one group of animals (Group I). These showed a gradual increase in calcium which reached a maximum about the last of October or the first of November (Table I and Text-fig. 1). This increase was fol-

lowed by a decrease which was succeeded in turn by a gradual and prolonged rise, terminating in a second maximum toward the end of January, 1927. A gradual fall in calcium then began and continued well into April. There was a suggestion of a secondary rise at the end of February and the first of March, but the third definite period of increase did not begin until the end of April or the first of May.

The variations in calcium shown by other groups of animals differed mainly with respect to the general level and the exact time of occurrence of maximum and minimum values. The conditions shown by Groups III and IV compare favorably with those of Group I (Tables III and IV and Text-fig. 1). The results for Groups III and IV are of especial interest in that they show that the period of increasing calcium which began in April or May continued through June, reaching a very high value on July 1. Unfortunately, no observations could be made on these animals during July and August, but by the middle of September, their calcium had again fallen to a point slightly below the mean normal level (15.6 mg.) or to values comparable with those for the preceding April and early May.

The interesting feature of the results for Group II is the extent of the variations shown (Table II and Text-fig. 1). The January-February maximum was considerably higher than that of Group I, while the March minimum was much lower. Moreover, the succeeding rise began while the calcium in the blood of other animals was still falling and well in advance of the rise in other groups.

Comparing the results for consecutive observations on animals caged in the laboratory (Groups I to IV) with those for animals examined as they were received from the dealer (Group V), it will be seen that, while the two sets of results agree with respect to the form of the variations, there is an interesting difference in the magnitude of the variations shown. In recently acquired animals, maximum values tended to be higher and minimum values lower, which suggests that the calcium content of the blood is subject to greater variation among animals living out of doors than among those caged in the laboratory.

The smoothed curves give the impression of a continuous or uninterrupted movement in a given direction which, with due allowance for technical inaccuracies and chance errors, is probably incorrect. A critical examination of the tabulated results will show that, in all prob-

ability, the prolonged rise and fall was accomplished by a series of short pendulum-like fluctuations with the general level rising or falling, as the case might be. This applies not only to calcium, but to all other values.

The situation presented by inorganic phosphorus is distinctly different from that of calcium (Tables I to V and Text-fig. 2). The striking feature of the results obtained for animals living under laboratory conditions is a marked and progressive decrease in inorganic phosphorus (Text-fig. 2); this is in sharp contrast with results for animals examined as they were received from the dealer. Available data for recently acquired animals show little or no change in inorganic phosphorus until January or February. There is a suggestion of a decrease in January and of a beginning rise in February. This rise was, however, comparatively slight and virtually ceased during March and April, to be resumed and continued through May and June.

Among the animals living under laboratory conditions, there are several features of the results that appear to be significant. Of these, the progressive decrease in inorganic phosphorus is most evident. It will be noted also that the results for each group of animals occupy a different level with comparatively little overlapping, and that the levels of successive groups rise in somewhat the same manner as the results for recently acquired animals examined at corresponding times (Text-fig. 2). Moreover, while all animals show a decrease in inorganic phosphorus, it will be seen that both the rate and extent of the decrease varied in different groups, and that in no case did the reduction proceed uninterrupted or at a uniform rate. In fact, each of the 4 groups of animals showed one or more periods during which the decrease in phosphorus ceased or gave place to an actual increase. This may be brought out by an analysis of the course of events in a given group of animals.

During November, the animals of Group I showed an abrupt increase in inorganic phosphorus extending over a period of 2 to 3 weeks with a return to the previous level about the first of December. This change coincided with a decrease and a subsequent rise in calcium. For 3 months thereafter, the decrease in phosphorus was comparatively slight, but during March and April it diminished at a more rapid rate, reaching the lowest level about the first of May. The last observation

on these animals gave a value that was higher than any that had been obtained since February which suggests that the phosphorus was again on the point of increase.

The distinctive feature of the results obtained for Group II is the marked decrease in inorganic phosphorus between January 5 and March 16, and the subsequent increase during March and April. In this instance, the change in inorganic phosphorus coincided with like changes in the calcium content of the blood.

The results for Groups III and IV show conditions that are again different. In the case of Group III, there was a reduction in inorganic phosphorus during the first 6 or 7 weeks; this was followed by an increase during March (Table III) and a second decrease which continued into June and was succeeded by a slight rise toward the end of June and the first of July. The striking feature of the change noted in Group IV is the marked reduction in inorganic phosphorus during the first 2 weeks of April. From this point on, the results compare favorably with those for Group III.

Considered as a whole, the results for inorganic phosphorus are somewhat confusing, due to the lack of agreement between recently acquired stocks and animals living under laboratory conditions, as well as to differences in the behavior of animals placed under observation at different times. Still, there is considerable evidence to show that inorganic phosphorus is subject to the action of influences which tend to raise or lower the level, as in the case of calcium, but these influences do not operate in precisely the same manner on animals confined in the laboratory and on those living out of doors. It is important to note that the change from outdoor to indoor life appears to be sufficient in most cases to initiate a decrease in the phosphorus content of the blood. In some instances, the immediate effect may be very slight, or even in the opposite direction, due perhaps to the fact that indoor conditions do not differ materially from those to which the animal has been accustomed (Group I); but, in other instances, the decrease in phosphorus may be marked (Group IV). Under such circumstances, the action of factors tending to increase the inorganic phosphorus of the blood may be completely obscured or accomplish no more than a reduction in the rate of decrease or a temporary stab-

ilization at a given level, such as occurred at several points in these experiments.

While it is reasonably certain that periodic variations in the trend of inorganic phosphorus occurred, definite movements can not be traced with the same degree of certainty as in the case of calcium. The indications are that there was a tendency toward lower values for animals of a given age, during the late fall and early winter months which was succeeded by a rise in January or February. The condition that prevailed during March and April is even more obscure, as some animals showed an accentuation of the decrease in inorganic phosphorus while others maintained a fairly high level. During May the trend was downward, and the final rise did not begin until June.

Applying the same principles of analyses to the values obtained for the sum and the product of calcium and inorganic phosphorus (Table VI and Text-figs. 3 and 4), it will be seen that, in general, these values reflect the variations shown by calcium and phosphorus respectively. This is particularly true of the sum, but the product shows a series of variations that is more clearly defined than those of phosphorus. In this case, the results obtained for recently acquired animals are of especial interest as they show a high value for February followed by much lower values for March and April and high values for May and June. In this respect, the results are in close agreement with those for calcium and for the sum of calcium and phosphorus.

The results for the ratio of calcium to inorganic phosphorus (Table VI and Text-fig. 5) disclose a condition that is almost the reverse of that shown by inorganic phosphorus. The general trend of values for all animals caged in the laboratory is upward, but variations in the absolute amounts of both calcium and phosphorus are reflected in these values.

Calcium-phosphorus ratio values for recently acquired animals show a gradual increase from October to February with a decided drop during March and April and a second slight rise during May and June. Animals in the laboratory show essentially the same conditions, if we disregard minor fluctuations. The chief difference between the two sets of values is that animals under continuous observation do not show a decided reduction in the ratio values for March and April, but a slight reduction or stabilization which is followed by a very

marked increase when the curve again turns upward. From observations on 2 groups of animals (Groups III and IV), it was found that the high values attained during this period of increase were eventually succeeded by a decrease. It is thus seen that, despite the progressive decrease in inorganic phosphorus, there is evidence of the occurrence of distinct periodic variations in the calcium-phosphorus equilibrium.

As a rule, the ratio of the product to the sum shows an inverse relation to the calcium-phosphorus ratio, and the sum or mean of the two values varies within very narrow limits or is virtually constant for a given set of conditions (1). An interesting exception to this rule is shown by the group of recently acquired animals (Table VI and Text-figs. 6 and 7). In this instance, it will be seen that the relation between values for the product-sum ratio and the calcium-phosphorus ratio is direct and not inverse. But, despite this difference, the quantitative relations are in close agreement with values obtained by repeated examination of given groups of animals, and these results give further evidence of the same series of periodic variations as was found by analysis of other values (Text-fig. 7).

The series of variations described above are, on the whole, so clearly shown by the curves that it would hardly seem necessary to resort to detailed statistical methods to determine whether variations of the order indicated represent actual differences in values or are within the range of probable variation due to random sampling. It is obvious that when a given change occurs gradually and by a series of pendulum-like swings, as in the present instance, nothing of importance can be learned by a comparison of successive values unless each value is based on a large series of observations. On the other hand, something may be gained by comparing values for given periods which represent a general trend or opposite phases of a supposed variation. A few comparisons of this kind will serve to indicate the probable significance of the results as a whole.

As the first example, we may compare the results obtained for calcium on Group I from November 12 to December 3 inclusive with those of December 17 to January 28 (Table I and Text-fig. 1). The mean values of all observations for these periods are 14.7 $\pm$ .128 and 15.9 $\pm$ .099 mg. respectively. As the difference of 1.2 $\pm$ .16 mg. is 7.5

times its probable error, it is certain that the difference between the values obtained for the two periods is significant.

There are even greater differences between results obtained for other groups during March and April, which in general represented a period of low calcium, and May, June, and July when the calcium rose to a much higher level. In the case of Group III, the mean value of all determinations from March 4 to April 27 inclusive is 15.0 ± .062 mg. and for May 11 to July 1, 16.7 ± .082 mg. which gives a difference of 1.7 ± .10. This difference is 17 times its probable error. The results for Group IV, comparing values for March 11 to April 1, and May 25 to July 1, are 15.1 ± .068 and 17.1 ± .073 mg. with a difference of 2.0 ± .10, which is 20 times its probable error. The March and April values for Group V give a mean of 15.0 ± .104 while the mean for May and June is 16.8 ± .180. In this case the difference of 1.8 ± .21 is 8.57 times the probable error.

Added significance is given to these results by the close agreement of the values obtained for different groups of animals at corresponding periods, as shown by the following tabulation of calcium values:

Group	Period of low values	Period of high values
III	15.0 ± .062	16.7 ± .082
IV	15.1 ± .068	17.1 ± .073
V	15.0 ± .104	16.8 ± .180

Less can be gained by a statistical analysis of the results for inorganic phosphorus. The progressive reduction in inorganic phosphorus among animals living under laboratory conditions is so evident that no further proof of the validity of this change is necessary. There are, however, a few instances in which a presumptive increase in inorganic phosphorus occurred in the course of the general downward movement, and the data bearing on these changes may be examined more critically. One of the best illustrations of a change of this kind is furnished by the increase in inorganic phosphorus which occurred in the animals of Group I during the middle of November. The observations for October 21 and 29 give a mean of 4.19 ± .099 mg. and those of November 12 and 19, 5.00 ± .078 mg. This difference is 6.43 times its probable error (.81 ± .126) and hence is definitely significant.

An analogous increase in inorganic phosphorus occurred in the animals of Group II between April 6 and May 11. The mean value for this period was 4.13 ± .075 mg. as compared with a mean of 3.88 ± .056 for March. As this difference (0.25 ± .09) is only 2.78 times its probable error, it may not be significant, but probably is.

A comparison of the values obtained for Group III on May 25 and June 8 with those for June 17 and July 1 gives a difference (0.48 ± .10) that is 4.8 times its probable error, and the October and November results for Group V compared with values for May and June give a difference (0.66 ± .18) that is 3.66 times the probable error. It is evident, therefore, that even in the case of inorganic phosphorus, there were instances in which a significant reversal of the dominant trend occurred.

Statistical comparisons of the results obtained for various measures of the relation between calcium and phosphorus have not been worked out in detail as it is evident that essentially the same situation obtains as in the case of calcium and phosphorus.

In this connection, however, some reference may be made to the degree of variation shown by the results obtained for individual animals at any given time as indicated by the coefficients of variation. With few exceptions, the coefficients of variation (Tables I to VI and Text-figs. 8 and 9) for consecutive determinations of both calcium and inorganic phosphorus are smaller than those for all observations on a given group of animals (1, 2) and they tend to diminish with successive observations. This, of course, indicates that the variation shown by the animals of a group at a particular time is less than that shown by the group during the course of the experiment and that group results tend to become more and more uniform.

There is, however, an evident relation between the variation of mean values and the magnitude of the coefficients. In general, it may be said that, so long as mean values remain comparatively constant, the coefficient of variation tends to be small, but the occurrence of either a decided increase or decrease in mean values is usually associated with an increase in the coefficient of variation. If the change in mean values is gradual and continuous, the coefficient tends to diminish until the magnitude of the mean values approaches the extreme limits of normal when the coefficient again increases. Further

more, while exceptionally high or low values may have a large coefficient of variation, it will be seen, by reference to Tables I to V, that if a high or low value is maintained, the coefficient is usually small. This is especially true of calcium.

From what has been said, it will be seen that there is no inherent relation between the magnitude of a mean value with respect to normal and its coefficient of variation. The relation found is one that indicates the occurrence of change, and in this sense the coefficient of variation becomes a valuable index of the stability of the animal organism with respect to particular conditions. Thus, a large coefficient or an increase in the coefficient of variation for a given series of calcium determinations indicates not only an uncertainty as to the true mean value, but is clearly indicative of an unstable condition and suggests the probable occurrence of a change, while a small coefficient or a decrease in the coefficient implies stability and uniformity of action.

The most important deduction to be drawn from the analysis of these results with respect to time, is that both the calcium and the inorganic phosphorus in the blood of normal rabbits are subject to progressive and periodic variations which effect not only the absolute but the relative amounts, or the equilibrium between the two substances which, within the limits of normal, is probably of more importance than absolute amounts. In like manner, periodic variations are more important in the present connection, than progressive variations as they provide a better basis for a study of any relation that may exist between changes in the chemical composition of the blood and susceptibility to disease.

The results reported agree with observations originally made by Hess and Lundagen (3) on calcium and inorganic phosphorus in the blood of infants and young children and with the results obtained by Grant and Gates (4) for calcium in the blood of normal rabbits, in so far as the occurrence of variations in one or the other of these substances is concerned. There are, however, certain differences. The variations found by Hess and Lundagen were confined largely to inorganic phosphorus, and no significant variations were found in adults. Moreover, there was a steady reduction in phosphorus from December to March

which was followed by a rise during May and June producing what was termed by them a seasonal tide.

In the experiments of Grant and Gates, the results for inorganic phosphorus were not analyzed. The highest values for calcium were obtained in May and November and the lowest in January, but no results were reported for February, June, July, or August.

In the experiments reported above, periodic variations were more clearly defined in the case of calcium than inorganic phosphorus, but the results differ from those of Grant and Gates as to the time of occurrence of maximum and minimum values. The results for inorganic phosphorus are more difficult to interpret, but it seems that for recently acquired stocks, the lowest value was obtained in January and that the increase began in February. There was, however, little or no further increase during March and April, while the highest value was obtained in June. Among animals living in the laboratory, no period of maximum or minimum values can be fixed for reasons which have been stated above. In one case (Group II) a clearly defined minimum occurred in March and this was succeeded by a definite rise; in other cases, the lowest values occurred in May and during the 1st week of June. And these were succeeded by a slight rise.

Differences such as those cited above may be of no particular importance. At any rate, there is no reason to assume that the course of events in man and animal should agree in all respects, or that variations in the calcium and inorganic phosphorus content of the blood of animals should follow a perfectly uniform course with respect to time. On the contrary, from what is known concerning analogous variations in organic constitution (5) and susceptibility to disease (6), it is to be expected that both the time of occurrence and the extent of any change in the chemical composition of the blood would vary from year to year. Moreover, it has been found that qualitative and quantitative differences will occur even among groups of animals that are under observation at the same time, so that one must reckon with variations in the response of animals, as well as with variations in the action of causative factors.

The point to be emphasized is that systematic or orderly variations in blood calcium and inorganic phosphorus do occur; in some instances,

it may be the calcium; in others, the phosphorus that is affected, or both substances may be affected in varying degrees. In the final analysis, however, it is the effect of these changes on the calcium-phosphorus equilibrium which concerns us most, and while various expressions of this relation may show greater or less change than either calcium or phosphorus, the relations do vary in much the same manner as the absolute amounts of the two substances.

Periodic variations in blood calcium and inorganic phosphorus have been referred to as seasonal variations. Hess and Lundagen attributed the increase and decrease of inorganic phosphorus in the blood of infants to changes in the available amount of ultra-violet radiation but they recognized the possibility that other factors might contribute to the production of these conditions. There is abundant evidence to show that ultra-violet rays exercise a profound influence on the inorganic phosphorus content of the blood of both man and animals, and that certain phases of calcium metabolism are likewise affected but analogous effects can be produced by other means (dietary).

The results obtained in these experiments could be explained in part as light effects. This is particularly true of the changes that occurred in inorganic phosphorus. On the assumption that the inorganic phosphorus in the blood is determined to a large extent by ultra-violet rays of short wave-length, one could account for the variations observed among animals living out of doors and for the progressive decrease that occurred when they were brought into the laboratory and thus deprived of this type of radiation. Among animals living in the laboratory, it would be difficult, however, to account for periodic increases in inorganic phosphorus or even variations in the rate of decrease as an effect of short wave-length ultra-violet radiation, and an explanation of the results obtained for calcium would be even more difficult.

Increasing age and the inactivity incident to cage life might also account for a part of the progressive changes in both calcium and inorganic phosphorus, but not for periodic variations. Moreover, as the rate of decrease in phosphorus, with respect to time, was decidedly irregular, it is not likely that age and cage life *per se* were decisive factors in the production of these changes.

Progressive changes in the chemical composition of the blood might be accounted for in various ways, but under the conditions of these experiments, no satisfactory explanation can be offered for the periodic variations. It is evident that the occurrence of these changes is referable to some condition of life which varies roughly with the seasons and is capable of exerting its influence on animals living indoors as well as out of doors, but not necessarily to an equal degree or in precisely the same manner. It is highly probable that ultra-violet rays, in a positive or negative way, played an important rôle in the production of the results reported, but it is practically certain that this was not the only factor concerned. Stated in a general way, it may be said that, while a number of factors probably contributed to the production of the variations observed, the indications are that the light environment, including visible as well as ultra-violet rays, played an important rôle.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of 5 groups of normal animals over periods of 4 to 8 months. The material included animals from recently acquired stocks as well as animals that had been living under laboratory conditions for long periods of time. The results were analyzed with especial reference to the occurrence of periodic and progressive variations in the absolute amounts of calcium and inorganic phosphorus and the relations between the two substances.

It was found that, among animals living in the laboratory, both calcium and inorganic phosphorus, as well as all expressions of the equilibrium between the two substances, exhibited a definite tendency to a progressive increase or decrease, as the case might be, and that clearly defined periodic variations occurred in all classes of animals. The progressive change was most marked in the case of inorganic phosphorus, the periodic change in the case of calcium, while both conditions were clearly shown by various expressions of the relation between calcium and inorganic phosphorus.

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THE RÔLE OF CLASMATOCYTES AND CONNECTIVE TISSUE CELLS IN NON-SPECIFIC LOCAL CUTANEOUS IMMUNITY TO STAPHYLOCOCCUS.

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Plates 29 to 32.

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In 1923, Besredka (1–4) stated that he could produce a specific generalized immunity in rabbits and guinea pigs to the subcutaneous or intracutaneous injection of staphylococcus and streptococcus by the injection of specific broth filtrates, or, by the local application to the skin of specific broth dressings.

The immunization was best produced in two ways: (1) by the intracutaneous injections over a small area of either (*a*) a sterile specific broth filtrate (the organism was grown 10–14 days in broth, filtered and the filtrate reinoculated with the same organism and grown again for 14 days and again filtered) or (*b*) of a vaccine made by heating a 24 hour broth culture of the specific organism at 60° for 30 minutes; (2) by the application for 24 hours over a limited skin area of compresses moistened by either the sterile specific broth filtrate or the heated killed broth vaccine.

The reaction to a subcutaneous injection of 1–2 cc. of a 24 hour broth culture in the control animal was a large sloughing ulcer, whereas in the protected animal, there was only a small localized abscess. Others (5) repeated these experiments with similar results. Gratia (6), however, obtained the same results with broth. By intracutaneous injection, Mallory and Marble (7) produced a local cutaneous immunity to staphylococcus in rabbits with sterile broth as well as with the broth filtrate. Miller (8) protected guinea pigs against staphylococcus with specific broth filtrates, vaccines, horse serum, broth and concentrated meat broth, all of these substances being injected intracutaneously or applied as compresses. The immunity was well localized, spreading but slightly beyond the area treated and in no sense considered specific. Since Besredka's work, clinicians have treated infections of various kinds with specific broth filtrates or vaccines in broth. Citron and Picard (9, 10) review the work done by others and claim to have had good

results themselves in the treatment of erysipelas, furunculosis, carbuncles and osteomyelitis. They used polyvalent broth filtrates and take for granted that the action is specific.

The aim of the following experiments was (1) to investigate the effect of local broth compresses, (2) to determine how constant this protection was and (3) to endeavor to throw some light on the mechanism of the protection.

Method.

Staphylococcus aureus (our laboratory number 35), isolated from a case of human osteomyelitis, was grown on agar for 48 hours and suspended in saline just before injections. In a few experiments, the staphylococcus was grown in broth. In any one experiment, the suspension from all the tubes, obtained by adding 2 cc. of normal saline to each tube, was pooled and a sample of this standardized roughly by centrifugation in a Hopkins tube for 5 minutes at 2000 revolutions per minute (odometer). It was found that a dose of .015–.06 cc. of bacteria suspended in 3 cc. of saline when injected subcutaneously in the abdominal wall or shoulder of a guinea pig produced, in over 66.1 per cent of the control animals, the characteristic sloughing lesion to be described later. The same dose was used in all the animals of any one experiment, and in all instances, the suspension was shaken immediately before the animals were injected. The control and "compressed" animals were injected alternately.

The organism was grown in broth for 10–14 days, filtered through a Berkefeld filter, the filtrate reinoculated for 14 days and refiltered to produce Besredka's filtrate.

Compresses were made of 6–8 layers of gauze, large enough to cover the abdomen and held in place by adhesive strips for 48 hours previous to the injection. They were kept moistened by frequent applications of the various substances used.

Compresses were the only method of protection used. All the experiments were on guinea pigs weighing from 210–250 gm. The broth used in poulticing was the usual laboratory beef extract broth (3 gm. Liebig's Beef Extract, 5 gm. sodium chloride, 10 gm. peptone (Fairchild's), 1000 cc. distilled water—boiled 1 hour, filtered, titrated to pH 7.6 and sterilized).

The Lesion.

The injection of the standard dose of bacteria subcutaneously in the abdominal wall caused death in 23 of the 121 control animals. 80 of the remaining 98 showed a diffuse cystic swelling covering the entire abdomen which appeared during the first 24 hours. The ani-

mals were sluggish and did not take nourishment. Redness, induration and pain were conspicuously absent at this time. During the next 48–72 hours, the edges of this swelling became firm, indurated, red, painful and warm to the touch. The skin in the center became discolored and frequently broke open exuding a serosanguineous material. In 5–7 days, the whole central area sloughed, leaving an ulcer, 2–3 cm. or more in diameter, with raised indurated edges and a granulating base. Healing took place in 3–4 weeks. This lesion we called 3 plus (+++).

Compresses of broth or filtrate applied to the shaved abdominal wall for 48 hours made the skin wrinkled, thick and slightly boggy. 24 hours after the usual subcutaneous injection of *Staphylococcus aureus*, there was a diffuse induration, red and painful, quite in contrast to the soft cystic swelling in the control animal. There was little general reaction as evidenced by activity or feeding. In most of the animals, a small localized swelling, $\frac{1}{4}$–$1\frac{1}{2}$ cm. in diameter, appeared by the 3rd or 4th day. This eventually discharged thick creamy pus or healed by resolution. This lesion is called 1 plus (+). In many of these cases, the lesion was a minute nodule containing a drop of pus.

In some of the animals the abscess formed was much larger and ran longitudinally up and down the midline, with thick indurated edges. After several days, this broke down discharging creamy pus. This lesion is called 2 plus (++).

To recapitulate, the large ulcerative lesion is called 3 plus (+++), the long abscess 2 plus (++) and the nodule or localized abscess 1 plus (+).

RESULTS.

Experiments show that ordinary meat broth is just as effective in altering the control reaction as the specific filtrate (Experiments 16a and 70). Furthermore, dry compresses and compresses moistened with water or saline give some protection, but it is not as complete as that given by broth (Experiments 19 and 64a).

The protection obtained was localized, for when animals were compressed with broth on the abdomen for 48 hours and then injected elsewhere in the shoulder region as in our experiments, the reaction

was as great in these animals as it was in the controls that had no compresses (Experiments 11a, 16b, 64b).

To determine if the protection afforded by broth was constant, a number of experiments were done (Nos. 18, 30, 34, 35, 38, 39, 41, 58, 66, 71). Of 70 controls, 9 died, while 51 of the remaining 61 showed large ulcerative lesions (3 plus, +++). Of 66 treated with broth prior to injection, 4 died; while of the remaining 62, only 2 showed a 3 plus (+++) lesion; 3, a 2 plus (++) lesion; and 57 had small localized abscesses. If all experiments with broth are totaled, of 121 controls, 23 (19.0 per cent) died and 80 (66.1 per cent) had 3 plus (+++) ulcerations. Among the 116 broth "compressed" animals, there were 7 deaths (6.0 per cent), while 98 (84.4 per cent) had localized abscesses.

To determine how long immunity lasted, animals were injected at various intervals after removal of the broth compresses. Although the experiments were not conclusive, there was evidence that the protection lasted more than 24 hours and less than 7 days.

It is evident from these experiments that guinea pigs can be protected in a fairly constant manner against the subcutaneous injection of *Staphylococcus aureus* by the local application of broth compresses.

In order to observe the histological changes accompanying the foregoing phenomena, control animals and animals which had been treated with broth compresses for 48 hours were killed at various intervals after bacterial injection and sections were taken of the abdominal wall. Animals previously injected with trypan blue intraperitoneally (once daily with 3 cc. of a 1 per cent solution for 4 days) were similarly treated. In all, 218 guinea pigs were used for histological study.

Sections included (1) normal abdominal wall, (2) abdominal wall after 48 hours of broth compressing, (3) the abdominal wall from control and "compressed" animals at varying intervals after bacterial injection (6–9 hours, 18–30 hours, 72 hours, 96 hours, 120 hours, 6 days and 10 days), (4) duplicate sections from animals previously injected with trypan blue.

The animals were killed by injecting formalin into the heart and the specimens obtained were washed and fixed in the usual manner before being imbedded in paraffin. All sections were stained with hematoxylin and eosin. In addition from the specimens vitally stained with trypan blue, one section was mounted unstained and one was faintly counterstained with carmine.

Normal Skin.

The normal skin of the guinea pig presented no unusual features. The epidermis was approximately 2–4 cells in thickness, the corium had the usual compact papillary and lower reticular layers. Of most interest for our purpose was the narrow reticular zone of subcutis contained between the hair follicles and the striated muscle layer. In this layer, were distinguished the two chief types of cells found in the connective tissue—the elongated connective tissue type with a large elliptical, compact, dark staining nucleus and a small amount of cytoplasm and the clasmatocyte with an irregular, indefinite outline, a large eccentric nucleus and cytoplasm filled with vacuoles of various sizes. The latter cells, the clasmatocytes, were often clustered at nodal points in the reticulum. They took up trypan blue in the vacuoles. A few dye-containing cells were scattered through the corium, but the majority were in the subcutis.

After Broth Compress for 48 Hours.

After an animal had been "compressed" with broth for 48 hours, the demonstrable changes were: (1) thickening of the epidermis, (2) edema of the subcutis, (3) a striking proliferation of clasmatocytes and connective tissue cells in the subcutis and (4) a moderate exudation of small mononuclear and polymorphonuclear leucocytes.

The epidermis was definitely thickened, comprising 4–8 cell layers. There was an edema of both corium and subcutis, most striking in the latter, for while in the normal animal it was only about one-fifth of the width of the corium, now it had become of equal breadth. All through the skin there was an increased number of cells most marked in the subcutis. While there was a moderate number of polymorphonuclears and small mononuclear leucocytes, there was, especially in the subcutis, a marked increase in the number and size of the clasmatocytes and the elongated connective tissue cells. The tissue macrophages were often of enormous size with a very definite outline that was more rounded in this than in the resting stage. A large vesicular nucleus rather poor in chromatin was pushed eccentrically by the rich vacuolization of the cytoplasm. Occasionally they contained two nuclei. There was no striking evidence of mitosis in any section. Trypan blue was taken up in large amounts by the tissue macrophages and segregated and concentrated in the vacuoles. Although the cells were most dense in the subcutis, a fairly even distribution through the corium might be taken as evidence of motility. The clasmatocytes were enmeshed in a whorl of connective tissue cells

which were also distinctly increased in number and size. There were many small mononuclear cells of various sizes and shapes, grading from the smallest with a compact, eccentric, often bean-shaped nucleus and clear cytoplasm to larger cells with a more vesiculated nucleus, whose cytoplaasm often contained a few vacuoles. The blood vessels of the lower corium were dilated and contained polymorphonuclear leucocytes, monoculcear cells and red cells. The endothelium was swollen and showed slight signs of proliferation. Occasionally around the capillary was found a cluster of small mononuclear cells.

The reaction of the skin to a broth compress took place largely in the subcutis as an edema accompanied by a striking increase of clasmatocytes and connective tissue cells. There was also a distinct thickening of the epidermis.

6–9 Hours after Bacterial Injection.

(*a*) *Controls.*—The control animals presented a tremendous edema of all layers of the skin, extending down through the connective tissue muscle sheaths. The epidermis was thinned, the papillæ flattened and the hair follicles compressed. Polymorphonuclear leucocytes were scattered throughout the corium and subcutis in large numbers. They were filled with bacteria, but showed definite signs of degeneration, *i.e.*, haziness of cell outlines, pycnosis, and fragmentation of the nuclei. There were relatively few mononuclear cells, most of which were small. Clasmatocytes were scarcely to be found, a fact which was confirmed in the trypan blue specimens which showed that very few cells ingested the dye. Connective tissue cells were not increased in number. Scattered through the edema, were many extravasated red blood cells. At this stage there were very few extracellular bacteria.

(*b*) *Broth-Protected.*—In the animals treated with broth compresses, the histological picture was quite different. The epidermis was still more thickened, the papillæ were prominent and the hair follicles well preserved. Edema was present throughout, although not as marked as in the control animals. The cellular infiltration, however, was much denser, particularly in the subcutis. Polymorphonuclear leucocytes predominated. They were filled with bacteria, but showed no signs of degeneration as evidenced by staining reactions. Small mononuclear cells of the types previously described were present in

very large numbers. There were many clasmatocytes scattered through and at the periphery of the exudate. The number of fibroblasts was increased so definitely as to suggest beginning organization even at this early stage in the lesion. In the trypan blue sections, the clasmatocytes at the periphery of the lesion took up the dye in large amounts, while in the most central part of the exudate, these macrophages were often filled with bacteria and phagocyted blood cells, so that the dye previously ingested was scattered through the cell. The blood vessels were dilated and congested and the swollen endothelium showed signs of proliferation. The differences between the control and the broth animals were found chiefly in the subcutis. In the broth-protected animal there was (1) a proliferation of clasmatocytes, (2) a proliferation of fibroblasts with beginning organization and (3) the preservation of the integrity of the polymorphonuclear leucocytes. There was also a marked thickening of the epidermis.

21-30 Hours after Bacterial Injection.

(*a*) *Controls.*—In the control animals, the epidermis showed signs of compression, often being lifted from the corium. The whole corium stained poorly and was disintegrating. The edema, still marked throughout the subcutis, had not increased, but the polymorphonuclears present showed further signs of degeneration. There was no attempt at organization. In the trypan blue sections, practically no cells took the dye. The epidermis, however, was stained diffusely, a sign of necrosis. There were few extracellular bacteria.

(*b*) *Broth-Protected.*—In the broth-prepared animal, the epidermis was still further increased in thickness. The edema was decreased, but the cell exudation was richer in the subcutis. A definite zone of proliferating fibroblasts was beginning to circumscribe the lesion. Polymorphonuclear leucocytes, increased in number, still retained their definite cell outlines and nuclear delineations. Among the fibroblasts were large numbers of clasmatocytes with richly vacuolated cytoplasm, often enormous in size and occasionally having two nuclei. Trypan blue was found abundantly in these cells. Many clasmatocytes had phagocyted bacteria, polymorphonuclear leucocytes and red blood corpuscles and showed only scattered dye granules. The blood

vessels were full, the endothlium swollen and proliferating and frequently endothelial lining cells jutted into the lumen of the vessel.

Rapidly advancing organization due to proliferating fibroblasts, together with marked activity of the clasmatocytes which were beginning to act as scavengers were the most marked changes at the end of 21–30 hours.

48–72 Hours after Bacterial Injection.

(*a*) *Controls.*—The control animal showed a marked degeneration of epidermis and corium. In the subcutis, the edema had decreased with no increase in cellular infiltration. The polymorphonuclears showed advanced signs of disintegration and bacteria, which previously were practically all intracellular, were now found in large numbers outside of the cells. Frequently large bacterial masses were seen, suggesting *in vivo* multiplication. Beginning signs of organization were seen in the lower part of the subcutis. In this zone, when the specimen had been stained with trypan blue, occasional cells took up the dye.

(*b*) *Broth-Protected.*—The broth animals showed a little greater thickening of the epidermis with broad deep papillæ. In the corium, there was a moderate cell increase, while in the subcutis there was a definitely walled off abscess. The zone of organization composed of fibroblasts and macrophages was thick. A large number of the cells in the peripheral portion of the exudate was enclosed in macrophages which were often engorged with phagocyted polymorphonuclears and debris. This was further shown by the fact that in the trypan blue specimens, few cells, except those at the extreme periphery, ingested the dye in large amounts, while it was present in many cells as scattered granules. The number of smaller mononuclear cells was decreased, while the number of large mononuclears was increased.

4–10 Days after Bacterial Injection.

(*a*) *Controls.*—In the control animals, the corium and epidermis had sloughed, leaving an ulcer with an organizing base just above the partially or wholly disintegrated muscle layer. In this zone, many cells now showed some avidity for trypan blue granules.

(*b*) *Broth-Prepared.*—In the broth-prepared animal, the abscess had definitely localized itself in the subcutis. Eventually, it either came to the surface and was evacuated or the clasmatocytes took up the cellular exudate and ingested it, while the fibroblasts proliferated through the lesion and organized it. At the end of 10 days, the clasmatocytes had largely digested their burden and the cytoplasm again showed marked vacuolization, so that the trypan blue was distributed in large amounts in the vacuoles.

To summarize, broth causes a marked stimulation of the cells of the subcutis, the clasmatocytes and the connective tissue cells, which seemed to protect the animal against the overwhelming effects of the bacteria. The macrophages phagocyted bacteria, thus relieving the burden imposed upon the polymorphonuclears so that they in turn were not destroyed by the infection. In addition, the clasmatocytes later phagocyted the polymorphonuclear leucocytes before they could disintegrate and free the bacteria. Concomitant with this was the rapid proliferation of fibroblasts walling off the lesion. The origin of the smaller mononuclear cells is difficult to determine. However, it was evident that they may develop into larger phagocytic cells and thus contribute largely to the number of these cells seen in the subacute stage of the lesion.

COMMENT.

Inasmuch as there is no question of specificity involved in these experiments, it remains to correlate the histological changes, *i.e.*, the increase of clasmatocytes, fibroblasts and round cells, with the marked reduction in mortality and the definite alteration of the inflammatory reaction.

While it is generally recognized that the tissue macrophages play a part in the subacute stages of inflammation, it has not been emphasized until recently, that they can offer an effective barrier to bacterial infection.

Metchnikoff (11) and his pupils considered the large mononuclear leucocytes of the blood as the typical macrophages and thought that the large cells in the connective tissue were of lesser importance. In experimental streptococcus infection (12), they believed that the phagocytes of the connective tissue had nothing to do with the disposal of bacteria except in so far as they engulfed polymorphonuclear leucocytes which contained them.

In experimental inflammation of the subcutaneous tissue, both aseptic and bacterial, Maximow (13, 14) definitely separated the clasmatocytes from the fibroblasts by their marked difference in motility and phagocytic activity. Macrophages which were present in large numbers at an early stage in the inflammation, especially the aseptic lesion, he thought came from three sources, (1) through proliferation of fixed tissue macrophages, *i.e.*, clasmatocytes, (2) through the development from the polyblasts or small mononuclears present in the tissue and (3) by the development from lymphocytes and mononuclear cells coming in from the blood stream.

Tschaschin (15) confirmed these findings in experimental peritonitis. Many others (for full reference, see Gay (16)) have observed the early appearance of large phagocytic cells in peritoneal inflammation. Following the work of Evans and Scott (17) who established definite vital staining reactions for clasmatocytes as opposed to the connective tissue cell, it was determined that these phagocytic cells in the peritoneal exudate were of connective tissue origin (Cunningham (18)).

While Rous and Jones (19) and Smith, Willis and Lewis (20) showed that in tissue culture, clasmatocytes have marked phagocytic activity, it remained for Gay and Morrison (21) and Gay and Clark (22) to definitely link these cells with immunity. They found that substances such as infusion broth or diluted egg white, locally injected, produced a marked increase in the number of clasmatocytes in the pleural cavity of rabbits. Such an animal was thus protected against many times the fatal infective dose of streptococcus when injected intrapleurally. In the normal animal, the streptococci increased until the death of the animal (5–7 days), while in the broth-prepared animal, the cultures from the pleural cavity were sterile in from 3–4 hours. Substances such as aleuronat which produced largely an increase in polymorphonuclear leucocytes, gave no such protection. After bacterial injection, rabbits actively or passively immunized against streptococci showed a much earlier mobilization of clasmatocytes in the pleural cavity than control animals.

We cannot discuss the much disputed relationship between clasmatocytes, large mononuclears of the blood and endothelial cells. However, we believe that the large phagocytic cells seen after broth compresses are proliferated largely from the clasmatocytes of the connective tissue. Morphologically, they are similar, their staining reaction the same and their rapid development into large cells with richly vacuolated cytoplasm is different from the gradual change of the small mononuclear cells into phagocytic cells even under the added stimulus of bacteria. There is no doubt that these latter cells contribute to the number of macrophages later seen in the lesion. Furthermore, broth compresses cause a concomitant proliferation of the elongated connective tissue cells. This is evidence of the stimulation of the connective tissue cell as a whole.

The rapid increase in the number of fibroblasts plays an important part in the protective reaction. Their function would seem to complement the activity of the clasmatocytes. The latter phagocyte the bacteria early, while the fibroblasts organizing around the focus hinder the spread of noxious material before it can be taken up by the phagocytic cells.

Polymorphonuclear leucocytes and small mononuclear cells certainly play a part later in the reaction. But these cell types, however, are too few in number directly after broth compressing and before bacterial injection for one to imagine that they are the effective agents in increasing the resistance.

After bacterial injection, the early disintegration of the polymorphonuclears in the control animal is of importance. This allows a proliferation of the organisms and a recrudescence of their activity before the clasmatocytes are present in large enough number to assist by ingesting the degenerating leucocytes. We infer that the presence of a large number of clasmatocytes previous to injection in the broth animal not only diminishes the virulence of the bacterial attack by the phagocytosis of the organisms, but by ingesting the leucocytes containing staphylococci, they prevent a recurrence of bacterial activity.

SUMMARY AND CONCLUSION.

1. Plain broth is just as effective as specific broth filtrate if used as a skin compress for the protection of guinea pigs against a subcutaneous injection of *Staphylococcus aureus*.

2. Plain broth compresses applied for 48 hours previous to bacterial injection sometimes prevent the death of the animal and practically always alter the inflammatory reactions.

3. This protection is not specific and is localized to the area "compressed."

4. The protection lasts at least 24 hours after removal of the compress.

5. Broth compresses applied to the abdominal wall of a guinea pig for 48 hours produced definite histological changes, especially in the subcutis, *i.e.*, edema, proliferation of clasmatocytes, thickening of the epidermis together with a moderate exudation of polymorphonuclears and small mononuclear cells.

6. The histological response to the subcutaneous injection of staphylococci was different in the control and the broth-prepared animal.

7. In the broth-prepared animal, there was an increase in clasmatocytes and fibroblasts with a dense exudation of polymorphonuclears, which latter, in the main, did not degenerate. The clasmatocytes phagocyted bacteria early and later engulfed the polymorphonuclears, while the fibroblasts rapidly walled off the lesion. The result was a localized abscess which either came to the surface and ruptured or was absorbed and organized.

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EXPLANATION OF PLATES.

PLATE 29.

All sections described have been stained with hematoxylin and eosin.

FIG. 1. Section through normal abdominal wall. Low power.

FIG. 2. Section through abdominal wall after broth compresses had been applied for 48 hours. Low power.

FIG. 3. Section through abdominal wall of control animal (not "compressed") 48 hours after bacterial injection. Low power.

FIG. 4. Section through abdominal wall of broth "compressed" animal 48 hours after bacterial injection. Low power.

FIG. 5. Section through abdominal wall of control animal (not "compressed") 96 hours after bacterial injection. Low power.

FIG. 6. Section through abdominal wall of broth "compressed" animal 96 hours after bacterial injection. Low power.

FIG. 7. Section through abdominal wall of control animal (not "compressed") 6 days after bacterial injection, showing ulceration. Low power.

FIG. 8. Section through abdominal wall of broth "compressed" animal 6 days after bacterial injection. Low power.

PLATE 30.

FIG. 9. Section taken after broth compresses had been applied for 48 hours. Shows clasmatocytes, fibroblasts and mononuclear cells. Oil × about 475.

FIG. 10. Control animal (not "compressed") 8 hours after bacterial injection. Shows degeneration of exudate cells. × about 325.

FIG. 11. Broth "compressed" animal 8 hours after bacterial injection. Shows exudate cells well preserved and a proliferation of fibroblasts. × about 325.

FIG. 12. Same as Fig. 10. Oil × about 475.

PLATE 31.

FIG. 13. Same as Fig. 11. Shows clasmatocytes containing bacteria. Oil × about 475.

FIG. 14. Animal (not "compressed") 21 hours after bacterial injection. Shows marked degeneration of exudate cells. × about 325.

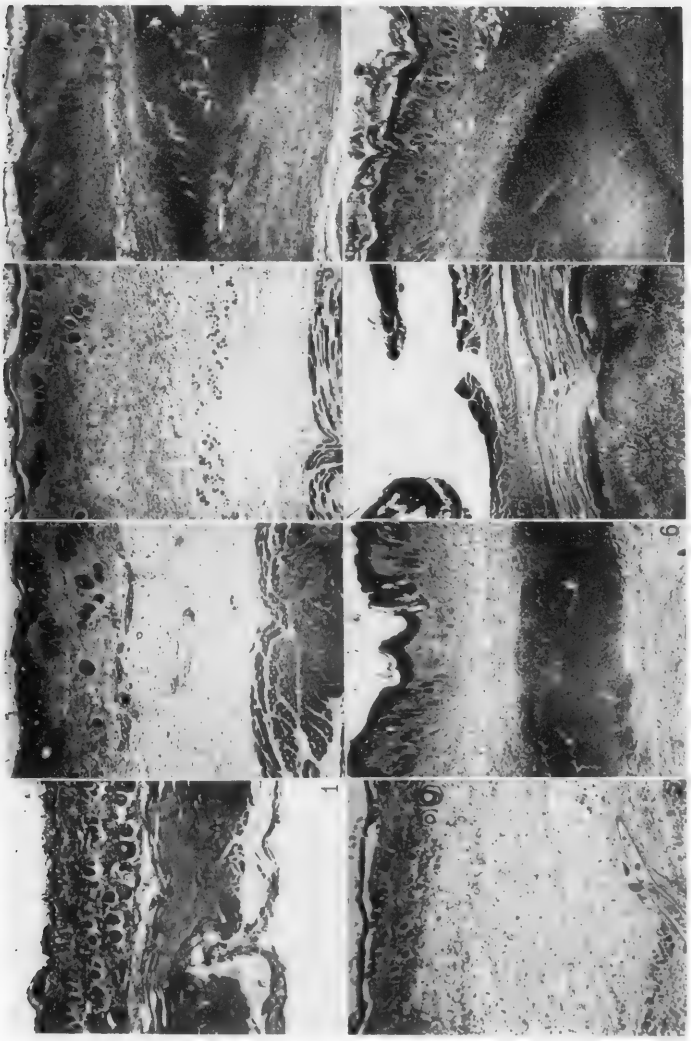
FIG. 15. Broth "compressed" animal 21 hours after bacterial injection. Shows many clasmatocytes, fibroblasts, small mononuclear cells. Cells of exudate well preserved. × about 325.

FIG. 16. Same as Fig. 15. Oil × about 475.

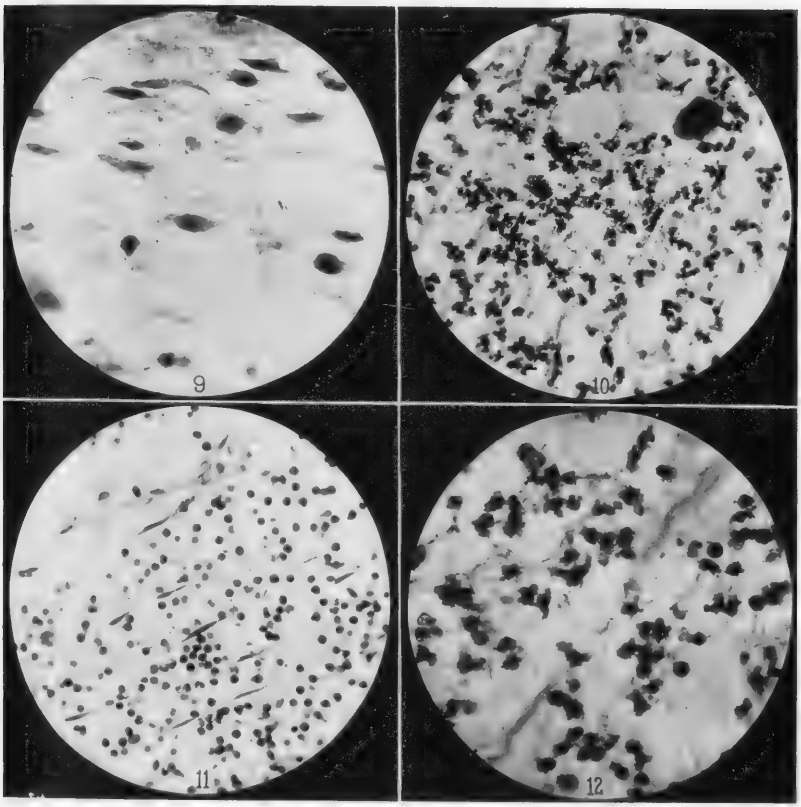
PLATE 32.

FIG. 17. Control animal (not "compressed") 72 hours after bacterial injection. Shows marked cellular degeneration and extracellular bacterial proliferation. Oil × about 950.

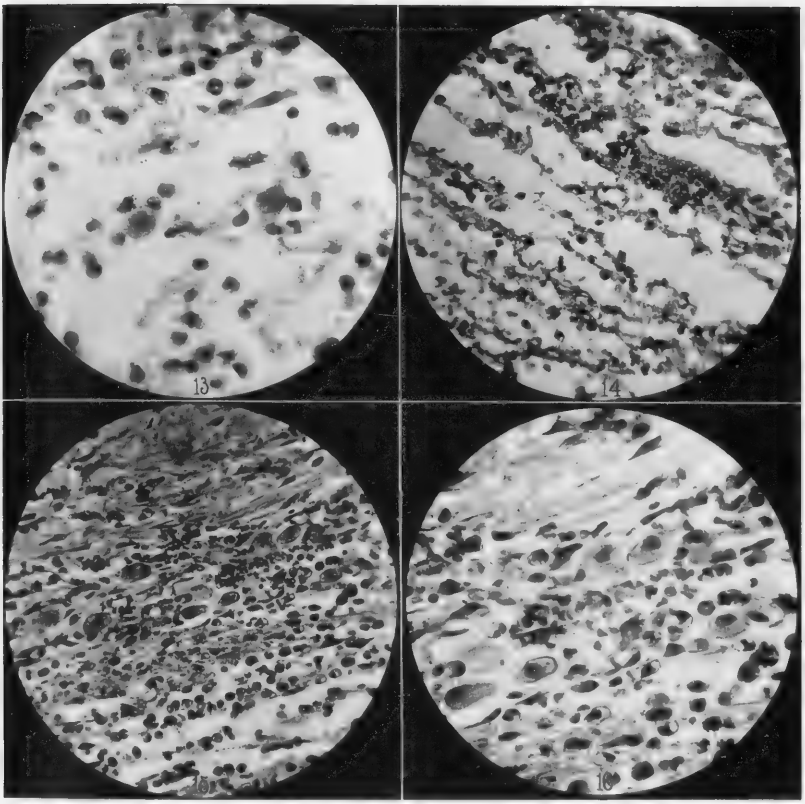
FIG. 18. Broth "compressed" animal 72 hours after bacterial injection. Shows phagocytosis of the cells of the exudate by macrophages. Oil × about 950.



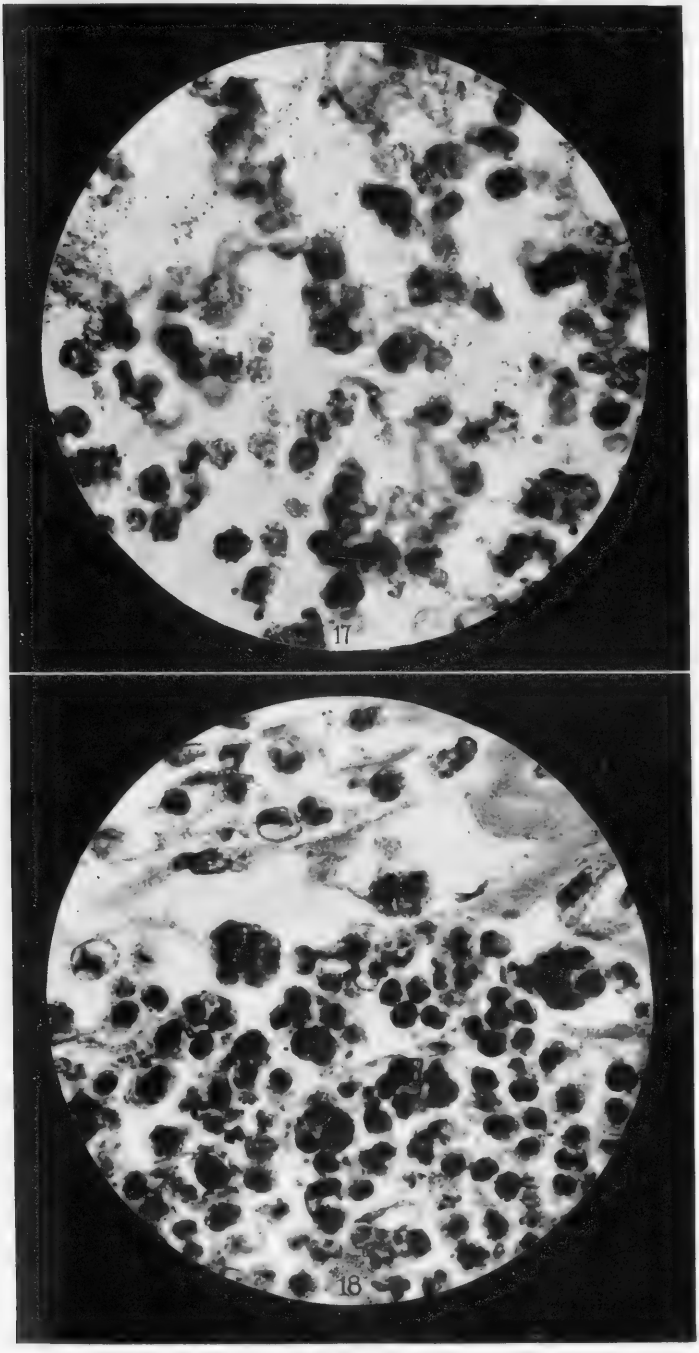
(Freedlander and Toomey: Local cutaneous immunity to staphylococcus.)



(Freedlander and Toomey: Local cutaneous immunity to staphylococcus.)



(Freedlander and Toomey: Local cutaneous immunity to staphylococcus.)



(Freedlander and Toomey: Local cutaneous immunity to staphylococcus.)

THE ELECTROPHORESIS OF THE BLOOD PLATELETS OF THE HORSE WITH REFERENCE TO THEIR ORIGIN AND TO THROMBUS FORMATION.

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Little is known of the physicochemical characteristics of blood platelets. There has been much dispute concerning their origin. The study of their cataphoresis affords a means of determining a fairly definite physicochemical constant which defines the make-up of these bodies in specific channels. Such data on the cataphoresis of the blood platelets of the horse and some other incidental observations, form the basis of this communication.

Review of the Literature Concerning Origin of Platelets.

The following review merely indicates the variance of opinion. It is incomplete.

In 1906 Wright (1) maintained that the cytoplasm of megacaryocytes was parent to that of blood platelets. Bunting (2) and Ogata (3) confirmed Wright's work. Brown (1913) (4), however, showed that hyperplastic endothelial cells in the marrow, and mononuclear and transitional cells in the marrow, spleen and blood, could also give rise to blood platelets.

Menne (5) made a specific immune serum from leucocytes and platelets and concluded from his studies that the structures of the leucocyte and platelet vary. Perroncito (6) (1920) believed that platelets arise from the red and white cells of the circulating blood. Schilling (7) (1921) came to the conclusion that the nuclei of red cells play a significant rôle in platelet formation. Erede (8) (1921) rejected the megacaryocyte origin. Rosenthal and Falkenheim (9) (1922) performed rather careful and well controlled experiments. They found that an erythrocytic immune serum was highly agglutinative for red cells but had a comparatively

* Work completed during the tenure of a Medical Fellowship of the National Research Council.

negligible influence on platelets. Platelet antisera also failed to agglutinate red cells. They concluded that, from the point of view of receptor structure, platelets and red cells present significant differences, while with the cells of the leucopoietic system the platelets possess a common receptor system indicating a common origin for the platelets. Marchesini (10) (1923) maintained that platelets arise from degenerated red blood cells which are phagocyted by megacaryocytes and then transformed by these cells into the form found in the blood. A rather complex hypothesis has been offered by Demel (11). This theory seems to hold for the origin of these cells (platelets) by a precipitation process direct from the blood plasma. The process is to be governed by megacaryocytes in the presence of physiological necessity. Further evidence in support of Wright's view was given by Katsunuma (12) (1925). Petri (13) in the same year denied the validity of Wright's views. Stahl, Horstman and Hilsnitz (14) by means of an iodine fixation method showed that certain granules were specific for platelets and megacaryocytes, again supporting Wright.

Method.

A modification of the Northrop (15) cataphoresis cell described elsewhere by Freundlich and Abramson (16) was used.

The plasma was oxalated by the addition of 8.5 cc. of a saturated solution of K oxalate per liter of horse blood. The platelets remain well preserved in the ice box for at least 48 hours.

The velocity of the platelets and of the polymorphonuclear leucocytes was determined as follows: The study of red cell migration at different levels in the cell permits the estimation of V_w, the velocity of the water in the midregions. The velocity of another particle in the midregions is then expressed by the equation

$$V_o - V_w = V \tag{1}$$

where V_o is the observed velocity of the particles and V is the absolute velocity. The reader is referred to previous communications for further data on method and related phenomena of cataphoresis (16, 19).

The Migration of Single Platelets.

The data of Table I are from six different horses. The mean velocity for the platelets is .45 μ per sec. per volt per cm. Polymorphonuclear leucocytes migrate with the same speed, within the limits of experimental error. In fact, simple observation confirms the measurements. Practically no difference in speed is observed. It may be recalled that in plasma lymphocytes migrate 15 to 30 per cent, and red cells about 90 per cent faster than leucocytes (19). The

same relationship holds therefore for blood platelets. Considering the difficulties of the method, the high conductivity of the medium and the low electrokinetic potential, with the exception of Plasma 2, the agreement in the five other specimens is excellent. It is surprising that blood platelets which are supposed to be so fragile retain for so long the same surface characteristics as far as the electrokinetic po-

TABLE I.

The Cataphoresis of Platelets in Plasma.

The speed of polymorphonuclear leucocytes is given in the last column. Although red cells and small lymphocytes have different velocities, note that platelets and polymorphonuclear leucocytes have the same velocity (six horses).

Plasma	Age	Platelets		Polymorphonuclear leucocytes
		V_o	V	V
	hrs.	*μ per sec. per volt per cm.*	*μ per sec. per volt per cm.*	*μ per sec. per volt per cm.*
1	30	.71	.41	.46
2	6	.82	.59	.57
	30	.76	.55	.60
3	6	.65	.46	.52
4	6	.57	.40	.43
5	6	.67	.51	.53
6	6	.68	.46	.54
	Mean excluding No. 2		.45	.49
	ζ potential (millivolts) (26.5 × μ per sec. per volt per cm.)		12	13

tential is determined by this surface. One is almost led to believe that their surface is determined by the presence of the plasma proteins rather than by an inherent composition.

The Migration of Aggregates of Platelets.

According to classical conceptions (17) the ζ potential of agglutinated blood platelets should be lower than that of single cells. In

specimens 30 hours old clumps of from 5 to about 20 platelets have been studied. There is no appreciable difference between the cataphoretic velocity of these aggregates and that of single cells. It is possible that some change in the ζ potential takes place incidental to the withdrawal of the blood. Just how far the aggregates observed represent a mechanism of slow coagulation by the particles below the critical potential must be reserved for future discussion.[1] The formation of aggregates without change in electrokinetic potential has been reported previously by Freundlich and Abramson for red cells (16).

Another outstanding feature of platelets is that they are able to stick to the glass walls of the cataphoresis cell with a remarkable tenacity. (That platelets are "sticky" has, of course, been noted hitherto.) This force is so great that a stream of water sucked through the cell does not remove them. The same adhesive quality has been discussed previously for leucocytes (19, 20). On the other hand, it is curious that neither red cells nor lymphocytes under the same conditions are possessed of similar properties. This fits in remarkably well with the behavior of all four types of cells in the presence of injury to tissue or capillary wall.[2]

DISCUSSION.

It would seem from the preceding data that the surface of polymorphonuclear leucocytes and that of blood platelets are similar. The electrokinetic potential is the same for both in a highly complex medium. This is all the more striking because of the fact that lymphocytes and red cells have a cataphoretic velocity which is unmistakably greater. Offhand one is inclined to believe that the platelets and leucocytes have a common leucopoietic origin. This would fit in quite nicely with the theory of Wright as follows: The blood platelets arise from the megacaryocytes which have in their

[1] Polymorphonuclear leucocytes and even quartz particles form aggregates whose cataphoretic velocity is the same as that of single particles with the same suspension. This "isopotential" agglutination will be discussed further in a future communication.

[2] This point is discussed in detail in an article by the author to be published (Abramson, H. A., in Alexander, J., Colloid chemistry, New York, ii).

turn been derived from myeloblastic cells (21); and the transition from myeloblast to leucocyte is, as far as concerns surface change, probably not a complex one. Still, one should accept this rather convincing evidence with a certain amount of hesitancy as it has been found in further experiments that quartz particles migrate with the same velocity in serum as leucocytes.[3] It has been also found that such quartz particles are influenced by slight traces of proteins (*e.g.* 10^{-7} gelatin solution lowers the ζ potential of quartz appreciably (18, 22)). Now, whether the white cells and blood platelets act like an inert particle, surrounding their naked protoplasm or cell membrane with a sheath of the protein in the medium, or whether they have acquired during their development the surface giving them their charge, is a question which is intimately bound with studies of surface adsorption and cataphoresis of these blood units. Experiments on the point have been started. At any rate, one can say that platelets and leucocytes have similar surfaces—certainly slightly different from lymphocytes and very different from red cells. And one may assume with a fairly reasonable degree of certainty that the unchanged relationship through the development of both types is strongly suggestive of a common origin.

The stickiness of the blood platelets has been noted. It must be remarked in concluding that the so called glass surface of the cataphoresis cell is really covered by a more or less complete layer of protein in the presence of even small protein concentrations (18). The platelets are really adherent to a protein film. The magnitude of this adhesive force has in general been described. It has been mentioned that aggregates are formed without changing the ζ potential. With this in mind, one may look upon thrombosis from the following point of view. Incidental to injury of the vessel wall, the adhesive force possessed by platelets is sufficient to permit them to remain attached to the wall in spite of the flow of blood rushing by. No change in electrokinetic potential is needed to establish a state of aggregation. Aggregation can probably occur in plasma without any measurable change in the ζ potential. The piling up of blood platelets can easily be explained

[3] The data and connected theory will be given in Abramson, H. A., *J. Gen. Physiol.*, 1928, xi, in press.

by the stickiness of the cells themselves, produced by adsorbed or inherent protein films. The same mechanical, simple conception may be applied to the subsequent addition of leucocytes to the thrombus and the attachment of fibrin strands.

SUMMARY AND CONCLUSIONS.

1. The cataphoretic velocity of blood platelets (horse) in plasma has been found to be between .40 and .51 μ per sec. per volt per cm. The mean velocity obtained from five horses is .45 μ per sec. per volt per cm.

2. The cataphoretic velocity of polymorphonuclear leucocytes in similar specimens is practically identical with that of the platelets. This is noteworthy because of the fact that lymphocytes and red cells have different speeds.

3. With spontaneous agglutination of platelets, white cells and red cells, there is no change in the cataphoretic velocity incidental to aggregation.

4. The possible surface composition of platelets and white cells is briefly discussed.

5. The bearing of these findings on the origin of blood platelets and the mechanism of thrombus formation is demonstrated.

I am indebted to Professor H. Freundlich for his criticisms and advice.

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THE MODE OF SPREAD OF A FRIEDLÄNDER BACILLUS-LIKE RESPIRATORY INFECTION OF MICE.

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PLATE 33.

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During observations of spontaneous *B. enteritidis* infection among special populations of mice (1, *a*), we noted the sudden appearance of a second disease which killed a great many of the animals. It arose first as an epidemic in late August, 1925, continuing in waves during the autumn and winter, and finally, in the spring, disappearing completely. A second similar outbreak occurred in August, 1926. The dead mice showed at autopsy bilateral pneumonia, and yielded in cultures from blood and lung an organism resembling Friedländer's bacillus.

The disease, in its sudden onset, high morbidity and mortality rates, its recurring outbreaks decreasing in severity, and its eventual disappearance, suggested somewhat human plague of the middle ages and the recent epidemic of influenza. For this reason, it was chosen for study, and its manifestations analyzed according to the experimental methods which we have employed in the investigation of mouse typhoid (1, *b*). The present paper describes the clinical course, pathology, and bacteriology of the disease, and records certain special studies of reactions between the bacteria and their host.

The literature on Friedländer bacillus-like infections, save for that relating to pneumonias of human beings, is scant and indefinite. Human case reports of Friedländer bacillus lesions of various organs, especially lesions situated near the upper respiratory tract, are numerous, however. Abel and Hallwachs (2) give references in which are described the recovery of this class of organisms from the atmosphere, earth, dust, water, and slime. Animal strains were reported by Pfeiffer in 1889 (guinea pigs (3)); Klein in 1889 and 1891 (guinea pigs and mice (4)); Weaver in 1897–99 (guinea pigs (5)); Skschivan in 1900, 1903 (rats (6)); Perkins in 1901 (guinea pigs (7)); Sachs in 1902 (rats (8)); Schilling in 1902 (rats

(9)); Toyama in 1903 (rats (10)); Aujeszky in 1904 (rats (11)), and Xylander in 1906 (rats (12)). Xylander refers also to communications on the subject by Fricke, Wilde, Fasching, and Gaffky. In reading these reports, however, one receives the impression that the organisms described form a rather heterogeneous group of capsulated bacteria, the effects on animals of which, were in many instances, quite different from that associated with typical strains of Friedländer's bacillus.

Since 1912, little has been written on Friedländer bacillus infection of animals, except for two articles by Holman (1916 (13)) and Branch (1927 (14)), in which the spontaneous disease in guinea pigs was described.

The general features of the disease in mice, as observed by us, are as follows:

Clinical Course.—The incubation period is about 48 hours. When a few carriers are placed in contact with groups of 100 or more mice, a certain number dies within 5 days, while 50–70 per cent are dead within 2 weeks. An occasional animal with signs and symptoms of pneumonia recovers, while a small number seems to resist infection altogether. Similarly, when a culture of the bacilli is introduced artificially into the nares of mice, a few animals die on the 2nd day, 70 per cent by the end of the 1st week, and a small number survives as nasal carriers or appears entirely refractory.

Pathology.—In mice dying of this infection, the autopsy shows subserous, petechial hemorrhages, such as occur in other septicemic infections, and extensive inflammation of the respiratory tract. The lungs are inflated, uniformly red and moist, and the serosa is covered with a seropurulent exudate. On section the lung tissue is red and wet, contains little air, and the exuded fluid is viscid and "stringy."

The microscopic lesions vary in extent and degree. In most instances there is dilatation of the interstitial capillaries, with or without accompanying interstitial hemorrhage and edema. Many alveoli contain serum and, occasionally, red blood cells. Beneath the pleura the congestion and edema are especially prominent. In many cases there is a fibrinous cellular exudate over the surface endothelium. The more advanced specimens show perivascular, interstitial, and intraalveolar accumulations of mononuclear and a few polymorphonuclear cells. Blood vessel walls are infiltrated and generally surrounded by cells; bronchi and tubules appear normal.

When groups of mice are given small doses of the organisms (200–600 intranasally), the pulmonary lesions described above are reproduced, and the differences observed are found to be related directly to the duration of life of the animals inoculated. For instance, mice dying 48 hours after the instillation show perivascular accumulations of large numbers of bacilli, perivascular round cell infiltration, and interstitial congestion; while those surviving 4 to 5 days show fewer bacteria, diffuse interstitial congestion and edema, and accumulations of leucocytes. After a longer period the alveoli are filled with leucocytes.

The pneumonic condition is in general similar to that described by Branch in spontaneous guinea pig infections (14) and by Stillman and Branch in experimental Friedländer infection in mice (15). The nature of the lesions studied by us suggests that the inoculated bacilli reach the lungs by way of the blood stream rather than by the bronchi (1, *d*).

Bacteriology.—The Friedländer bacillus-like organisms may be cultured from the nasal passages, lungs, and blood. Except in cases of severe septicemia, they are not found in the intestinal tract. Under dark-field illumination they appear as large, non-motile, homogeneous, blunt rods. They are Gram-negative; the capsules stain vividly,

TABLE I.

Cross-Agglutination Tests Made by Dr. Julianelle with His A, B, C, and X Friedländer Strains.

Strain	Friedländer type sera: dilution 1:5					Mouse Strain 1. Serum 1:5
	Type A	Type B	Type C	Group X I	Group X II	
Mouse Strain 1	−	−	−	−	−	+++
" " 2	−	−	−	−	−	+++
" " 3	−	−	−	−	−	+++
" " 4	−	−	−	−	−	+++
" " 5	−	−	−	−	−	+++
Friedländer Bacillus "A"	+++	−	−	−	−	−
" " "B"	−	+++	−	−	−	−
" " "C"	−	−	+++	−	−	−
Group X I	−	−	−	+++	−	−
" X II	−	−	−	−	+++	−

and Wright's blood stain exhibits a large enveloping material surrounding a deeper staining substance. Dextrose, maltose, xylose, salicin, and mannite are fermented in 24 hours; saccharose in 3 to 4 days, and lactose in 7 to 12 days. Milk is acidified; no indol is formed; nitrates are reduced to nitrites.

The serologic reactions of the strains obtained by us are practically identical. Sera obtained from rabbits by injecting dead and living cultures agglutinated all strains to a maximum titer of 1:640. No antigenic relationship was established with other known strains of Friedländer's bacillus.

A series of cross-agglutinations and thread tests with five of our mouse strains and with Friedländer's bacilli, Type A, B, C, and X, was made for us by Dr. Julianelle (16). Table I gives the results. The five type sera of Friedländer's bacillus failed to agglutinate any of the mouse strains, and the mouse strain serum agglutinated none of Julianelle's five type strains of Friedländer's bacillus.

Colonies of the bacteria growing on agar plates from direct mouse autopsy culture are large, moist, and viscid, "stringing" easily on the platinum loop (Figs. 1 and 2). Bacilli from young, single cell colonies show no mucous material. At a later period, the entire colony, save for the peripheral zone of activity dividing bacteria, is composed of the enlarged cells, which in mass present a mucoid appearance.

Growth requirements of these organisms are relatively simple. No enriching substances, carbohydrates, serum, "X", or vitamine factors need be added to plain infusion agar or broth, pH 7.0–7.4. However, considerable sensitivity to temperature changes exists.

Experiment 1 compares the growth of cultures at 37° and 23°C.

Two series of tubes containing 5 cc. of plain infusion broth of pH 7.4 were inoculated with different dilutions, 10^{-1} to 10^{-9}, of a 48 hour broth culture of Mouse Strain 3. One series was incubated at 37°; the other left at room temperature, 23°. Growth in the two series was compared at 24 and 48 hours. 3 days later the cultures were examined by dark-field illumination, by staining methods, and by replating. 6 days after inoculation, the numbers of viable bacteria in each tube of the series were counted by the plating method.

The first four tubes of the 37° series showed good growth at 24 hours; the remaining five, 10^{-5} to 10^{-9} inclusive, were sterile. The entire series grown at 22° were turbid at 24 hours. The original culture used for inoculation contained about 800,000,000 organisms. Hence, as the findings show, an inoculation of 100,000 organisms was required to produce growth at 37°, whereas the smallest number, less than 10, grew at 22°. No further growth occurred at 48 hours. At this time, examination of cultures by dark-field and staining methods showed autolysis, great swelling, and reduction in numbers of bacilli in the tubes of the 37° series; the 22° cultures appeared normal. Counts at 6 days showed less than 100 bacilli per tube in the first four dilutions of the 37° series; the rest were sterile. Plates of tubes of the 22° series averaged about 170,000,000 organisms per cc. per tube.

The tests were repeated with other strains with essentially the same results. Hence, the optimal growth temperature is considerably less than 37°.

The next experiment was planned to ascertain the duration of life of these organisms in fluid culture.

Experiment 2.—Four flasks with 300 cc. of broth in each were inoculated with a 48 hour culture of Strain 1. Two received about 12,000 organisms and two about 10; actual counts were made immediately. One flask of each dilution was then incubated at 37°; the two others were left at room temperature, 22°. Counts were made of the numbers of bacteria in each flask at 3, 6, 25, and 52 hours, and at 10 months. The results are shown in Table II.

TABLE II.

Growth of Small Numbers of Friedländer-Like Bacilli at 37° and 23°C.

Time after inoculation	No. bacteria per cc.			
	Flask 1: inoculation 10^{-2} 22°	Flask 2: inoculation 10^{-2} 37°	Flask 3: inoculation 10^{-5} 22°	Flask 4: inoculation 10^{-5} 37°
hrs.				
0	12,000	15,000	10	10
3	48,000	9,000	0	0
6	70,000	3,000	30	0
25	180,000,000	0	18,000,000	0
52	325,000,000	0	280,000,000	0
mos.				
10	6,000,000*	0	5,000,000*	

* Rough variants 100 per cent. No mucoid forms.

Both incubated (37°) flasks were sterile at 24 hours and remained so. The two flasks standing at 22°, room temperature, showed good growth at 25 hours and viable organisms 10 months later. At this time, however, the mucoid colonies were replaced entirely by the rough variant colony type. Hence these cultures are found to be relatively simple in their requirements and able to survive for long periods of time at temperatures of about 20–24°C. Higher temperatures, however, prove harmful.

Colony type variation occurs readily with the Friedländer group of bacilli. Hadley and Julianelle have summarized the reports of early investigators. Hadley (17) found that rough colonies of Friedländer bacillus arise when cultures are grown for some days in broth or agar. Branch witnessed the same phenomenon in the cultures from guinea pigs (14). Julianelle noted transformations in his A, B, and

C type strains grown in antiserum (16). Our mouse cultures lose the mucoid colony appearance when grown in antiserum, and when kept 2 or more weeks in broth or agar. The variant colonies are non-mucoid, quite small, opaque, with regular margins and granular surfaces (Figs. 1, 2, 3).

The variant mouse strain colonies are not found under natural conditions. During the past $2\frac{1}{2}$ years, we have cultured the nasal passages of more than 2000 mice and autopsied at least as many more, and obtained only mucoid colony forms. We conclude, therefore, that the variant types do not occur in recognizable numbers during either the interepidemic or epidemic phase of the native mouse disease.

Single cell cultures from the variant colonies are relatively stabile in spite of various manipulations *in vitro* and *in vivo*, a reverse transformation from variant to the original mucoid form was not accomplished.

Bacilli from the variant colonies are smaller than those from the mucoid colonies, and lack the enveloping material; otherwise the two resemble each other. The short rods are Gram-negative and non-motile, fermenting the characteristic sugars, acidifying milk, and reducing nitrates to nitrites. They agglutinate in antimucoid sera to more than double titer. They are not flocculated by sera from Julianelle's Type A, B, and C strains.

Microbic Virulence and Host Susceptibility.

We have determined the effects of different numbers of bacilli on the amount and severity of the experimental infection, and have made titrations of the virulence of the same and different bacterial strains, over a period of more than 2 years.

Technique.—The general principles governing the technical procedures of these titrations have been described elsewhere (1, *b*). Briefly, we consider it essential to reproduce the natural conditions of infection as nearly as possible. The dosage, virulence, and host susceptibility tests are planned in such a way as to remove as far as possible disturbing variables. Thus, under given conditions, relatively constant results are secured. The circumstances most nearly reproducing the native infection are adopted as a standard. This

standard is then used for comparison for titrations of dosage, virulence, and host factors.

Cultures are grown in a uniform medium and treated alike throughout. Dosage is estimated in numbers of bacilli administered. The bacilli, suspended in a drop of salt solution, are introduced through a small glass pipette into the nasal passages of each mouse. The spasmodic inhalations of the animal aid the taking in of the fluid. Mice from the inbred Rockefeller Institute stock, raised under uniform environmental conditions, about 12 weeks of age, weighing 18–20 gm., and not previously exposed to the infection are used (1, *c*). The inoculated mice are placed in separate cages.

Standard Curve.—At the outset, the precision of the titration technique was determined by performing a number of titrations in duplicate and observing the amount of variation in the end results.

Technique.—Strain 1, obtained on October 25, 1926, from a mouse in one of the special populations, was used throughout. For each titration, a subculture in broth was made from the stock slant and grown for 48 hours at 23°. A dose of 200–600 bacteria per mouse was found to represent natural conditions most satisfactorily. Hence each animal was given intranasally, by glass capillary tube, 1 drop of a 1:10,000 0.85 per cent salt dilution. This volume contained 200–600 organisms, as checked each time by dilution plates. The mice were chosen according to the above specifications. After inoculation, each animal was numbered and placed in a separate jar. The entire number was then divided arbitrarily into groups of 20, 25, 50, or 100, according to the amount used, and the mortality in each group was compared. Dead mice were autopsied and cultured. The results of these tests are summarized in Table III.

On November 16, 1926, a titration was made with 50 mice, divided into two groups of twenty-five. Each group showed close agreement in amount and rate of death. 84 per cent were dead in each on the 18th day; none died thereafter (Table III). On November 22, a similar test was made, resulting in a final mortality on the 42nd day of 86 per cent, with a variation between groups of 6 per cent. A third titration on December 2 with three different doses and six groups of twenty mice each showed total mortalities of 92.5 per cent with a group variation of 2.5 per cent (1250 bacteria per mouse), 90 per cent total and group mortality (380 bacteria per mouse), and 55 per cent, with a variation between groups of 10 per cent (58 bacteria per mouse). On December 16, a fourth test with two groups of twenty and a dose of 195 bacilli resulted in the death of 70 per cent total and 5 per cent group variation mortality. A fifth titration on December 20, with two groups of twenty-five and a dose of 960 bacteria per mouse, showed a final mortality of 90 per cent with a 2 per cent variation of group mortality. On January 5, 1927, two groups of 50 each received 15,000 organisms per mouse, and two groups of 50 a dose of 115 bacilli. The mortalities at 42 days were 86 per

TABLE III.

Mortality during Days after Inoculation.

Date	Dose	Group	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	
11–16–26	250±	1– 25	2	4	3	8			2		2														21
		26– 50		6	8	3		1	1		2														21
		Total	2	10	11	11		1	3		4														42
11–22–26	350±	1– 25		8	3	2	2			2				1		1							1		20
		26– 50	1	10	3	4	2								2	1									23
		Total	1	18	6	6	4			2				1	2	2							1		43
12– 2–26	1250	1– 20	3	10	1	2		1		1								1							19
		21– 40		12	2			1	1			1	1												18
		Total	3	22	3	2		2	1	1		1	1					1							37
	380	1– 20	4	5	2	1			1	5															18
		21– 40	2	5	4	2	2		1	2															18
		Total	6	10	6	3	2		2	7															36
	58	1– 20		1	5			1		2															9
		21– 40		2	4	3			1	2							1								13
		Total		3	9	3		1	1	4							1								22
12–16–26	195	1– 20		4	5	1				1			1	1											13
		21– 40	1	5	6					1	2														15
		Total	1	9	11	1				2	2		1	1											28

12–20–26	960	1– 25	1	12	8	1																							22
		26– 50	2	9	9	2	1																						23
		Total	3	21	17	3	1																						45
1– 5–27	15,000	1– 50	1	25	12	2	2	1												1	1								45
		51–100		27	11	1		1												1									41
		Total	1	52	23	3	2	2												2	1								86
	115	1– 50		11	4											2	1		6										24
		51–100		12	4				1			1				2			10	3									33
		Total		23	8				1			1				4	1		16	3									57
1– 7–27	187	1– 50		23	13	2	1		2												1								42
		51–100		22	11	1	1		1												1								37
		Total		45	24	3	2		3												2								79

cent with a 4 per cent and 57 per cent with a 9 per cent final difference in group mortality.

The results of duplicate titrations, of which the above are a part, showed that the experimental technical error was insignificant; hence tests carried out as described above proved sufficiently accurate.[1] Furthermore, the mortality rate per day was found to be so uniform that average figures were taken to represent numerically the reaction of the Rockefeller Institute strain of mice to a dose of 200–800 bacteria of Strain 1. These total mortality percentages, when plotted against time in days, formed a frequency curve similar to those obtained in mouse typhoid studies (1, *b*) (Text-fig. 1). Hence, measurement of variations in dosage, virulence, and host susceptibility were compared

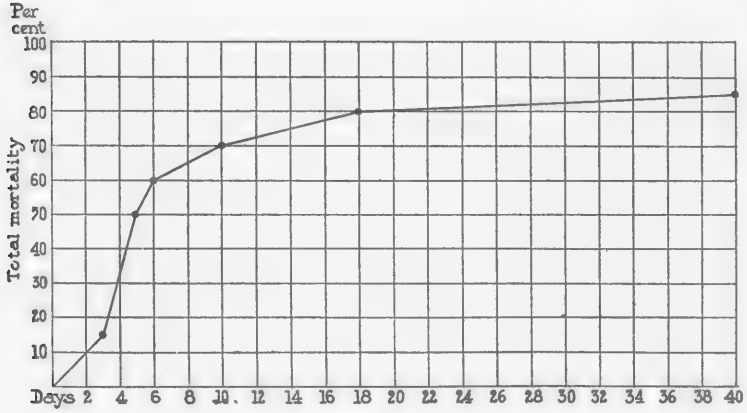
[1] Dr. John W. Gowen analyzed the data contained in Table III and commented upon it as follows: "Mathematically analyzed, using mortality rates and weighing them equally, the variation within the groups is quite insignificant in contrast to the variation between the groups made at different times, indicating that the technique has been satisfactory, thus:

Variance	Degrees of freedom	Mean square
Between groups	9	375.1 Z = .927 where for $P = .05$ Z = .56
Within groups	10	58.7

"Two possible causes of the significant variation between groups are obvious in the data. The first is variation in dosage, as indicated by the plate counts, and the second is seasonal variation, although the controlled environment of the mice and cultures make such an interpretation doubtful. The data are not well suited to attempts to separate these possibilities since the number of repeated titrations at different dates is not sufficient. However, if the material be drawn as a graph with death rate as one coordinate and dosage of bacteria as the other, it is noticed that for dosages from 58–400 there is a rapid almost linear rise in death rate from 55–390. At 400 bacteria the death rate becomes essentially the same to dosages up to 15,000. This suggests dosage as the important cause of the variation between groups. Approaching the problem slightly differently, there are two dates on which groups of mice were given different dosages, December 2, 1926, and January 5, 1927. Analyzing this data for influence of date of inoculation, the mean variance between dates is found to be 70 and within dates 433. Clearly the date of inoculation played an insignificant part in the death rate. As the cause of variation within the dates is the dosage, we may attribute to it the major rôle in causing the variation in the death rates within these data with influence of season or variation in technique as but minor and insignificant causes of variation."

either with these average figures or with those obtained from a control group tested simultaneously.

Dosage.—Observations of the spontaneous disease among the mice of the special populations indicated that contact with a very small number of the bacilli is fatal to most individuals. For example, surviving carriers in the mouse populations were found to harbor in their nasal passages but very few of the Friedländer bacilli, while mice from which fifty or more colonies were cultured invariably died within a few days. These findings were confirmed by actual titrations which showed



TEXT-FIG. 1.

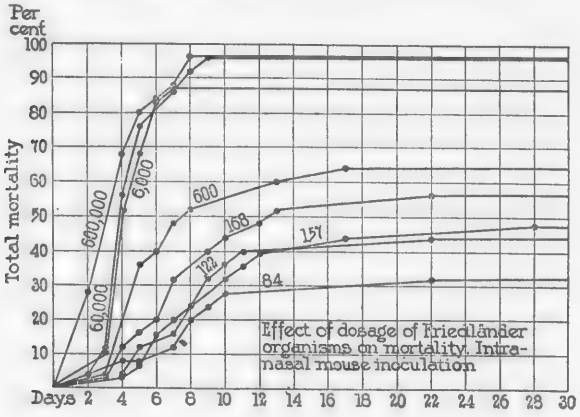
that while a small number of mice survived even a maximal dose, as few as 500 bacilli per mouse killed about 80 per cent within a week. Tests were made to determine this relationship more accurately.

Experiment 3.—Strain 1 was transferred from the stock slant to meat infusion broth, pH 7.4, and left at room temperature (23°) for 48 hours. Dilutions were then made in 0.85 per cent NaCl, counted by the plate method, and given intranasally to mice by means of a coarse capillary pipette. Eight dilutions were used; each was given to twenty-five mice. After inocuation, the animals were placed in separate jars. Mortality records were kept and autopsies done on all dead animals.

The duration of life of the individuals of each group is plotted in Text-fig. 2. The mortality rates of groups receiving 6,000, 60,000, and

600,000 bacilli were quite similar. No deaths occurred after the 9th day; one animal of the 600,000 and 60,000, and three of the 6000 group survived 30 days. In the remaining groups death rate and total mortality were related closely to dosage. 64 per cent of the group given 600 bacteria succumbed, 56 per cent of those given 168, 44 per cent of those given 122, and 32 per cent of those receiving 84.

These tests show that small doses (less than 100 bacilli), given to a group of twenty-five mice, are fatal to certain individuals; that doses ranging from this to over 1000 lead to a progressively increasing death rate and total mortality in groups of similar size; and that doses larger



TEXT-FIG. 2.

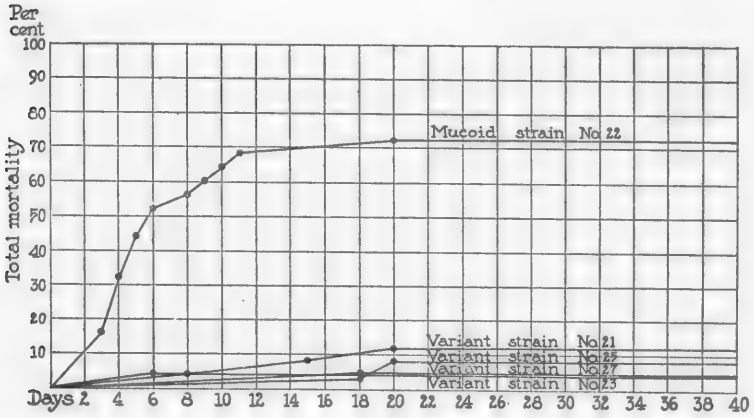
than 6000 organisms do not increase the number of deaths beyond a certain point. Regardless of the size of the dose, some individuals survive.

Virulence.—The virulence of the bacilli has been determined by tests similar to those described above. The standard dose of about 600 organisms was instilled intranasally. Sufficient animals were used to run each titration in duplicate. Strains of unknown pathogenicity were compared directly with known cultures, or indirectly by means of the standard curve described above (Text-fig. 1).

Repeated titrations of single strains over a period of 12 months showed no significant changes in virulence. The results of the tests

on Strain 1 are given in Table III. This culture has been kept on agar at 4°C. and transferred every 4 weeks. It was obtained from a mouse of a known susceptible race (1, *c*), 2 days before the onset of a severe spontaneous epidemic. The degree of virulence of the type-pure culture was found to remain relatively constant. Three other strains kept type-pure and tested in the same way showed insignificant fluctuations in virulence.

Rough variants obtained from old broth or agar cultures proved far less virulent than the parent mucoid type. The following test illustrates this difference.



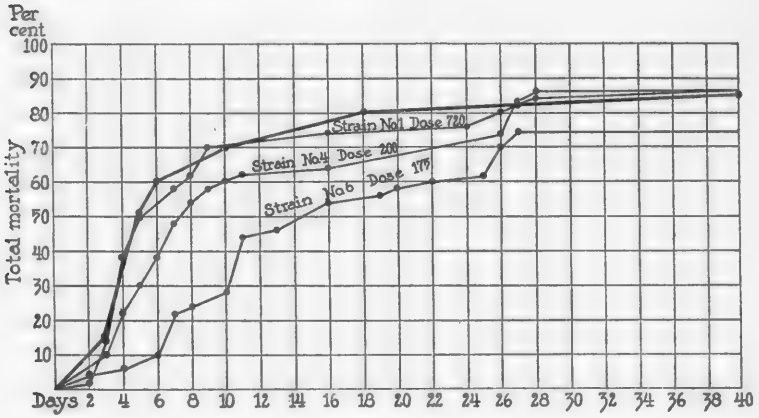
TEXT-FIG. 3.

A mucoid type strain, No. 22, obtained from a mouse of one of the special populations, September, 1925, its rough variant, No. 23, and three other rough variants from mucoid type strains isolated at about the same time were grown 48 hours in broth at 22°C., diluted 1:10,000, and administered intranasally to mice. Each culture was given to twenty-five individuals. The mortality record is plotted in Text-fig. 3.

The mucoid culture, No. 22, showed high virulence characteristic of the type, while its rough variant, No. 23, together with the three other rough strains, Nos. 21, 25, and 27, killed only very few of the animals—4.4, 12, 8, and 4.4 per cent respectively.

A number of titrations were made of cultures taken from mice of the

special populations in which the spontaneous infection was prevalent in endemic and epidemic form. The strains were chosen under varying conditions, with a view to determining differences in lethal power coincident with high or low population mortality, with cultivation of the bacilli in the circulating blood of susceptible individuals, and in the nasal passages of animals resisting infection. A chart describing the course of the infection among the special populations, with a record of the days on which these cultures were obtained, will be published later; at present it will suffice to state briefly the amount of disease prevailing at the time each culture was obtained.



TEXT-FIG. 4.

The method of titration of virulence described in a previous paragraph was employed throughout. Dilutions were made to insure a dosage of about 600 bacilli per mouse. Due to slight irregularities of growth, however, the actual counts as determined by plating varied considerably from this number. Such fluctuation in the relatively small doses of these organisms given has been shown to affect death rate considerably (Experiment 3), and it is responsible, we believe, for the irregulatities in some of the virulence curves.

The results of the virulence trials with unknown strains have been compared either with the standard control curve for Strain 1, or with a simultaneous control titration with Culture 1. Each group of mice

TABLE IV.

Mortality during Days after Inoculation.

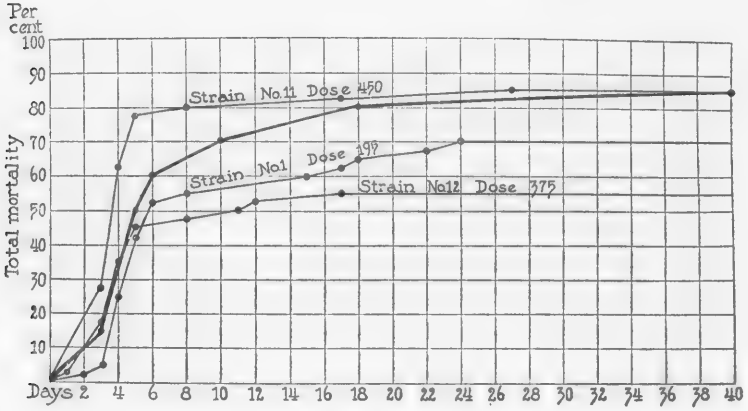
Date	Dose	Group	0–2	2	4	6	8	10	12	14	16	18	20	22	24	26	28–30	Total	Death rate	Average age at death	
11–22–26	720	1– 25			8	3	2	2			2				1		1	19	76	9.95±	1.07
Strain 1		26– 50		1	10	3	4	2								2	1	23	92	9.35±	1.04
		Total		1	18	6	6	4			2				1	2	2	42	84	9.61±	.75
	200	1– 25			8	4	4	1	1		1					2	2	23	92	11.17±	1.16
Strain 4		26– 50			3	4	4	2								3	4	20	80	14.90±	1.49
		Total			11	8	8	3	1		1					5	6	43	86	12.91±	.95
	175	1– 25				1	5		4	1	2		2			3	2	20	80	16.70±	1.12
Strain 6		26– 50		2	1	1	2	2	4		2			1		2		17	68	13.00±	1.17
		Total		2	1	2	7	2	8	1	4		2	1		5	2	37	74	15.00±	.83
12–16–26	450	1– 20			15	2	1											18	90	5.44±	.17
Strain 11		21– 40			10	4						1					1	16	80	7.88±	1.08
		Total			25	6	1					1					1	34	85	6.59±	.53
	195	1– 20			4	5	1				1			1	1			13	65	9.92±	1.26
Strain 1		21– 40		1	5	6					1	2						15	75	8.33±	.90
		Total		1	9	11	1				2	2		1	1			28	70	9.07±	.76
	375	1– 20		1	7	2			2			1						13	65	7.46±	.83
Strain 12		21– 40			6	2	1											9	45	5.89±	.31
		Total		1	13	4	1		2			1						22	55	6.82±	.52
12–20–26	960	1– 25		1	12	8	1											22	88	5.82±	.19
Strain 1		26– 50		2	9	9	2	1										23	92	6.22±	.26
		Total		3	21	17	3	1										45	90	6.02±	.16
	225	1– 25			6	11	4			1								22	88	7.18±	.31
Strain 13		26– 50			7	5	6											18	72	6.89±	.27
		Total			13	16	10			1								40	80	7.05±	.21
	580	1– 25			9	5	1	1	1	1								18	72	7.11±	.47
Strain 15		26– 50			11	5		1									1	18	72	7.22±	.87
		Total			20	10	1	2	1	1							1	36	72	7.17±	.49
1– 7–27	150	1– 25		1	6	7	2	8										24	96	7.83±	.36
Strain 17		26– 50			6	11	3	2										22	88	7.09±	.25
		Total		1	12	18	5	10										46	92	7.48±	.22

TABLE IV—*Concluded.*

Date	Dose	Group	0–2	2	4	6	8	10	12	14	16	18	20	22	24	26	28–30	Total	Death rate	Average age at death	
1–7–27	187	1– 50			23	13	2	1	2					1				42	84	6.76±	.34
Strain 1		51–100			22	11	1	1	1					1				37	74	6.57±	.36
		Total			45	24	3	2	3					2				79	79	6.67±	.25
	110	1– 25			9	5	1							1	1			17	68	8.06±	.97
Strain 16		26– 50			12	5	1			1								19	76	6.26±	.37
		Total			21	10	2			1				1	1			36	72	7.11±	.51
1–19–27	400	1– 50			14	17	6	3	1					1				42	84	7.43±	.33
Strain 18		51–100			3	8	4	6	4	2				1				28	56	9.93±	.49
		Total			17	25	10	9	5	2				2				70	70	8.43±	.29
	630	1– 50			7	9	7	4	3						1			31	62	8.68±	.47
Strain 19		51–100			5	19	3	4	1	1				1				34	68	8.24±	.40
		Total			12	28	10	8	4	1				1	1			65	65	8.45±	.31
2–28–27	500	1– 25			10	9		1		1								21	84	6.62±	.35
Strain 34		26– 50			12	8												20	80	5.80±	.15
		Total			22	17		1		1								41	82	6.22±	.20
	590	1– 25			9	8	2	1										20	80	6.50±	.25
Strain 19		26– 50			9	7	3					1	1					21	84	7.67±	.62
		Total			18	15	5	1				1	1					41	82	7.10±	.35
	320	1– 25			13	3	3											19	76	5.95±	.23
Strain 33		26– 50			10	5	1	1	1		1							19	76	7.11±	.50
		Total			23	8	4	1	1		1							38	76	6.53±	.28
	540	1– 25			12	3	1	1					1					18	72	6.67±	.54
Strain 32		26– 50			9	5	2	1										17	68	6.41±	.29
		Total			21	8	3	2					1					35	70	6.54±	.31
5– 2–27	420	1– 25			13	8	1											22	88	5.91±	.17
Strain 50		26– 50		1	8	6	3											18	72	6.22±	.26
		Total		1	21	14	4											40	80	6.05±	.15

receiving a given culture was divided arbitrarily into two subgroups of equal numbers, and the mortality of each compared with that of the total group. Hence a mean daily mortality figure was obtained and the extent of deviation from this mean of each subgroup noted.

Test 1. The Virulence of Freshly Isolated and Stock "Epidemic" Strains.— Strain 1 was obtained from the heart's blood of a susceptible Lathrop mouse, October 15, 1926, 2 days before the outbreak of a severe epidemic; Strain 4 from a similar animal, November 17, 1926, just prior to a similar wave. Strain 6 was isolated at the peak of an epidemic, August, 1925, from an individual of a different, though similar population. Since that time, it has been kept on plain agar at 4°C. On November 22, 1926, each strain was given to 50 mice which were then numbered and placed in separate jars. Two groups of twenty-five mice each were made arbitrarily for comparison of results. The dosage employed was as follows: Strain 1, 720 organisms per mouse; Strain 4, 200 organisms per mouse; Strain 6, 175 organisms per mouse. The results are summarized in Text-fig. 4 and Table IV.

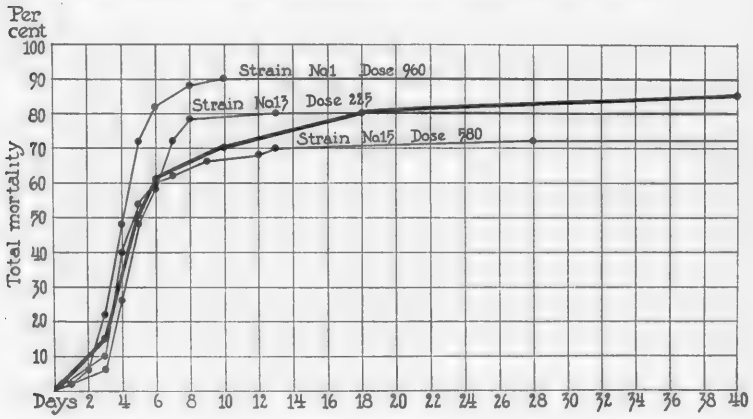


TEXT-FIG. 5.

The mortality of the groups receiving Strains 1 and 4 was similar throughout the period of the experiment. 84 and 86 per cent respectively, were dead on the 40th day. The final deviation from the mean of the subgroups was 8 and 6 per cent, and the average daily deviation 7.4 per cent for mice receiving Strain 1 and 8.6 per cent for those receiving Strain 4. The death rate of the group receiving Strain 6 was lower during the 1st week but deaths continued over a longer period of time. The final mortality was 74 per cent, with a deviation of subgroups from the mean of 6 per cent and an average daily deviation of 1.5 per cent. The mortality curves of the groups receiving Cultures 1 and 4 parallelled closely the standard mortality curve.

The two cultures, therefore, may be considered to be of equal virulence. Culture 6 seemed somewhat less effective, but this discrepancy is believed to be due to differences in actual dosage of bacilli received, rather than to any difference in their degree of virulence.

Test 2. The Virulence of Freshly Isolated "Epidemic" Strains Taken from the Nasal Passages of Healthy Mice.—Three strains were used in this test,—Strain 11 was obtained from the nasal passages of a healthy mouse of the so called "Friedländer" population 1 day prior to the outbreak of a severe epidemic, and Strain 12 from a similar mouse in the same population 1 week before the same outbreak. Strain 1 was again employed as control. Each culture was instilled into forty mice. The dose of Strain 1 proved to be 195 organisms per mouse, of Strain 11, 450, and Strain 12, 375. The results are shown in Text-fig. 5 and Table IV.

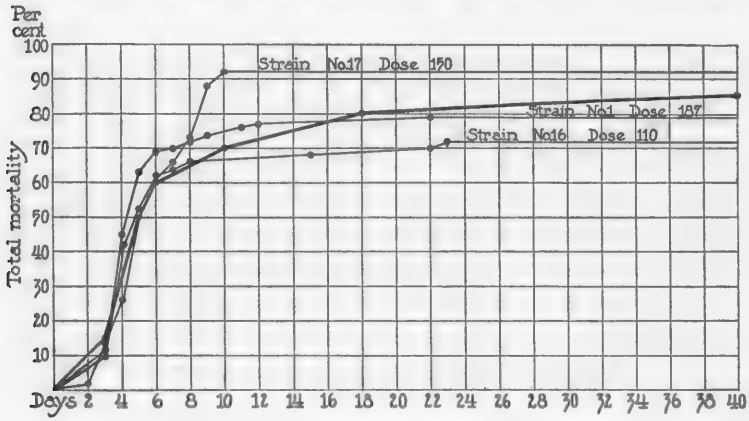


TEXT-FIG. 6.

The final mortality of the group receiving Strain 11 was 85 per cent with a deviation of the subgroups from the mean of 5 per cent and an average daily deviation of 7.4 per cent; that of the group receiving Strain 12 was 55 per cent, with a variation between groups of 10 per cent on the final day, and an average daily deviation of 4.9 per cent, while the final mortality of Strain 1 group was 70 per cent, with a 5 per cent variation and an average daily deviation of 5.6 per cent. The apparent low activity of Strain 12 was due, we believe, to undetermined differences in actual dosage received and not to a difference in virulence.

Test 3. The Virulence of "Epidemic" Strains Taken from the Nasal Passages of Surviving Mice.—Strains 13 and 15 came from individuals of the same population as Strains 11 and 12, and were obtained at the height of the epidemic mentioned above. The mice appeared healthy at the time the cultures were taken and survived the epidemic. Strain 1 was run at the same time as a control. Each culture was given to 50 mice. The dose of Strain 1 proved to be 960 organisms per mouse, of Strain 13, 225, and of Strain 15, 580. The results are summarized in Text-fig. 6 and Table IV.

The mortalities in all three groups were similar and approximated closely the figures of the standard mortality curve. 90 per cent of the Strain 1 group were dead on the 40th day, with the subgroups showing



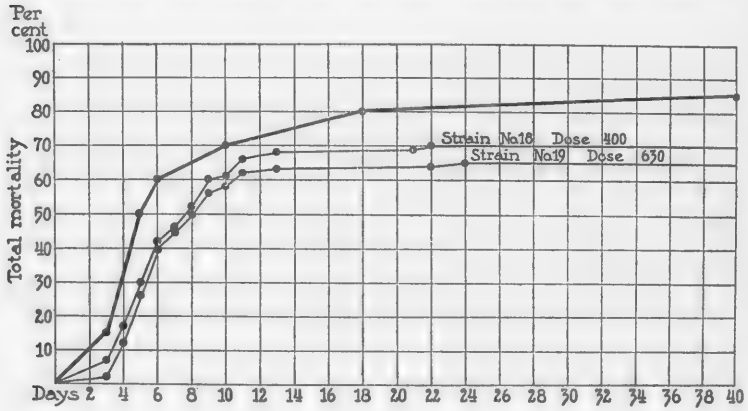
TEXT-FIG. 7.

a final deviation of 2 per cent and an average daily deviation of 2.2 per cent. The mortality of the group receiving Strain 13 was 80 per cent, subgroup variation 8 per cent, and an average daily deviation of 7.2 per cent, and that of the group given the No. 15 culture was 72 per cent, with subgroups identical at the end of the test and showing an average daily deviation of 1.3 per cent. The three cultures tested, therefore, may be considered as equally virulent.

Test 4. The Virulence of "Epidemic" Strains Taken from Heart's Blood of Infected Individuals.—Strain 16 came from a susceptible mouse of one of the special populations designated "G" population, at the height of a severe and long epidemic. Strain 17 came from a mouse of another special population designated

"Friedländer" population, also during the plateau of a severe and long spontaneous epidemic wave. Strain 1 was run simultaneously as a control. One hundred and eighty-seven bacilli of Strain 1 were given to each of 100 mice, 110 of Strain 16 to each of 50 mice, and 150 of Strain 17 to each of 50 mice. The results are shown graphically in Text-fig. 7 and Table IV.

The mortality curves of all three series are similar and follow quite closely the standard control curve. The final mortality of the Strain 1 group was 79 per cent, with a subgroup variation of 5 per cent and an average daily deviation of 4.3 per cent; of the Strain 16 group, 72 per cent, subgroup difference 4 per cent, and average daily deviation 5.2



TEXT-FIG. 8.

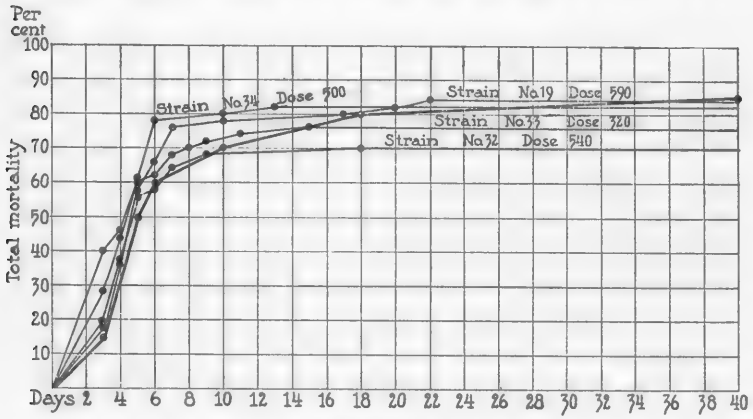
per cent, and of the Strain 17 group 92 per cent, subgroup variation 4 per cent, and average daily deviation 4.2 per cent. The three cultures therefore may be considered to possess equal virulence.

Test 5. The Virulence of "Epidemic" Strains from the Nasal Passages and Circulating Blood.—Strain 18 was obtained from the nasal passages of a healthy mouse of the special "G" population at the height of a severe epidemic. The individual from which this culture was taken survived. Strain 19 came from the blood of a mouse of "No. 6" population, dying at the height of a severe epidemic wave. Each culture was administered to 100 mice in doses of 400 and 630 organisms per mouse respectively. The results are charted in Text-fig. 8 and Table IV.

The mortality of each group was almost identical, and differed from that of the standard control curve by a small margin. The final

mortality of the No. 18 group was 70 per cent, subgroup difference, 14 per cent, average daily deviation 14 per cent; that of the No. 19 group was 65 per cent, with a subgroup variation of 3 per cent, and an average daily deviation of 2.9 per cent. For the instance of the large deviations in subgroups from the mean in the case of the No. 18 group, which is unique, we offer no present explanation. Nevertheless, these cultures may be considered equal to each other and to agree with Strain 1 in virulence.

Test 6. The Virulence of "Epidemic" and "Surviving" Strains.—Strain 19 was described in Test 5. Strains 32 and 33 came from the lungs of two mice of the



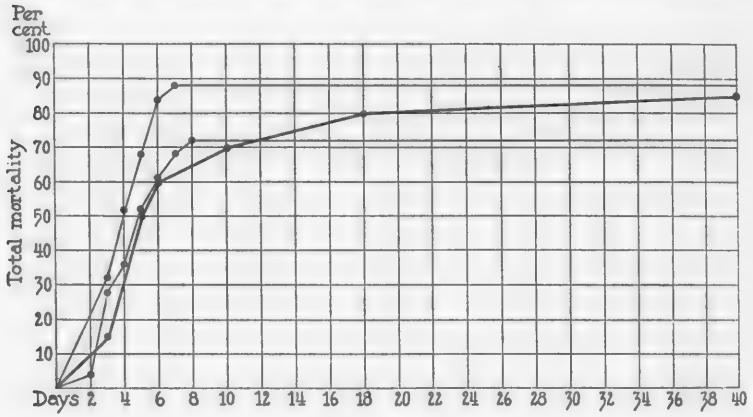
TEXT-FIG. 9.

"No. 6" population dying at the peak of a severe epidemic. Strain 34 came from the nasal passages of an individual of the same population, which had lived throughout the entire epidemic and was surviving and healthy in the interepidemic period at the time the culture was taken. Each strain was administered to 50 mice,—590 organisms per mouse of No. 19, 540 of No. 32, 320 of No. 33, and 500 of No. 34, the results of which are shown in Text-fig. 9 and Table IV.

The mortality curves resemble one another and the standard control curve of Strain 1. 82 per cent of the No. 19 group were dead on the 40th day. The final deviation of the subgroup was 4 per cent, and the average deviation 2.7 per cent per day. The final mortality of the No. 32 group was 70 per cent, with a final subgroup deviation of 2 per

cent and an average daily deviation of 1.5 per cent. That of the No. 33 group was 76 per cent, with no final difference in the subgroups and an average daily deviation of only 1 per cent; that of the No. 34 group was 82 per cent, with a final and average daily deviation of 2 per cent. These strains and Strain 1, then, are considered to be of the same virulence.

Test 7. The Virulence of a Surviving Culture, Strain 35.—This strain was obtained from the only carrier of the "No. 10" population, surviving a severe epidemic wave which had occurred 2 months previously. Within a week after the culture had been obtained from this mouse, it ceased to yield the Friedländer



TEXT-FIG. 10.

bacilli and at about the same time the disease disappeared entirely from the population. Two groups of twenty-five mice each were given the culture in a dose of 420 organisms per mouse. The results are shown in Text-fig. 10 and Table IV.

The mortality of each group is seen to approximate closely the figures obtained from the standard control curve. This culture, therefore, is considered to be of the same virulence as Strain 1 and all cultures previously tested.

The precision of the virulence titrations is ascribed to the fact that the experimental conditions were arranged so as to eliminate as many disturbing, unknown factors as possible, and to reproduce as nearly as possible natural conditions of infection. Hence the conclusion

seems justified that the virulence of type-pure mouse strains of Friedländer-like bacilli, with which we have dealt, remains constant under natural conditions.[2] Mucoid strains kept type-pure on agar do not

[2] Dr. Gowen analyzed the results of these virulence titrations and commented upon them as follows: "The results of Tests 1 to 7 inclusive may be analyzed for the influence of period in the epidemic at which the cultures were isolated in relation to death rate. This analysis is complicated by the effect of variation in dosage. There are at least two culture tests made for each group which will give some appreciation of this factor, however. Cultures before peak of epidemic were 1, 4, 11, and 12; at peak 6, 13, 15, 16, 17, 18, 19, 32, and 33, and in the inter-epidemic period were 34 and 50. The material is set out below:

Variance	Degrees of freedom	Sum of square	Mean square	
Between tests	18	2990	166.1	
Within tests	19	1384	72.8	Z = .412 where for P = .05 Z = .43
Total.....	37	4374		

"The variation between tests is greater than that within tests, as in the previous results for Strain 1. The significance of this difference is questionable, however. Z = but .41 while Z for P = .05 = .43. Furthermore, the variation between tests does not show the consistent relation when plotted against dosage of bacteria, that was previously shown for Strain 1. In fact, the death rates appear to be quite random so far as dosage is concerned.

"Two of the 18 degrees of freedom between tests are due to the point in the epidemic where the bacilli were isolated as indicated above. Dividing the data thus the variance and its associated constants are:

Variance	Degrees of freedom	Sum of square	Mean square	
Between epidemic points	2	150	75.0	
Within epidemic points	16	2840	177.5	Z = .431 where for P = .05 Z = 1.48
Total.....	18	2990		

"The analysis of the data on the epidemic point of bacterial isolation shows that the variation between epidemic points is much less than that within the different groups. The conclusion is consequently justified that the epidemic point of isolation of the bacteria plays no part in the total fatality of the epidemic it initiates in the Friedländer disease of mice. The differences within and between epidemic point groups are not significantly differentiated since Z = .43 while for P = .05 Z must be at least 1.48.

"The rapidity with which the organism kills for those animals dying offers another measure of the possible effect of period in the epidemic during which the organism was isolated. For the three groups as utilized above the average duration of life for those dying was for the preepidemic peak 8.14 ± .26;* peak of epi-

* Because of the skewness of the distributions these probable errors lose much of their meaning and must be interpreted with caution.

change in pathogenicity; strains obtained from animals before, during, and after epidemic periods, and in interepidemic times all agree in pathogenic activity, and strains taken from healthy carriers, from individuals acutely ill, and from animals dying of pneumonia and septicemia, are of the same high virulence. On the other hand, rough strains, developed in the laboratory, exhibit much lower virulence. The rough variant types were not observed to occur spontaneously at any phase of the natural infection.

DISCUSSION.

Throughout the studies of spontaneous Friedländer-like bacillus infection among special mouse populations, the conditions of experimentation were so controlled as to permit an analysis of the main factors determining the amount and spread of the disease. The cultures of Friedländer-like bacilli used in the present study were obtained from mice in these special populations, during various epidemic and endemic phases of the infection.

Hence, special attention is directed to the virulence titrations recorded in this paper, since they show that strains of bacilli derived from mice at epidemic and postepidemic periods, are of one type, of uniform and constant virulence, and in these respects conform to the observations made in earlier studies on mouse paratyphoid (1, *e*) and enteritidis (1, *a*) infections, and rabbit Pasteurella (1, *f*) infection.

The results of virulence determinations reported by us are not in

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demic group 8.11 ± .14; and interepidemic group 6.14 ± .12. Clearly there is a difference between the duration of life of the third and remaining groups. The bacteria of the interepidemic period appear to kill more rapidly and with less variation in time than those in the preepidemic or peak epidemic periods. The drawing of this conclusion does not appear to be justified, however, for if the data be examined, a clear association between the experimental groups is noted when these groups are arranged by dates. This association is largely due to the relatively long time which was taken to kill on the date of the first experiment and the short duration of life for the last two experiments. When the influence of the factor of time of experiment is removed the differences between the three groups of bacteria in the time with which they kill become insignificant, or there is no detectable influence of time of isolation of the bacteria and the duration of life for those mice which die."

accord with the views expressed by Topley, Lockhart, and Greenwood, in their papers on experimental epidemiology. These authors, it is true, have discussed the subject of virulence rather from the theoretical than from the experimental point of view, since they report few actual titrations of the mouse typhoid organisms with which they deal.

In order to make this divergence of attitude clear, we venture to review the papers of Topley and his associates at some length.

In 1919 (18), a series of tests of virulence, without control observations, is reported by Topley, in which mice of varied breeds, without known history affecting age, food, surroundings, and exposure to infection, were employed. Small numbers, all kept together in one cage, were used for each test. The culture employed was a stock strain of Pasteur Institute Danysz bacillus, fed on bits of bread. Survival of the animals following this procedure was, for the most part, random and unpredictable, and yet the varied effects were taken as indicating fluctuations in microbic virulence taking place during the experiments. No other possible factor seems to have been considered, and the results were cited as evidence in favor of the thesis stated in the earlier paragraphs of Topley's paper, that "an increase in the pathogenicity of the specific parasite is an essential factor in the rise of epidemics" (18).

That this view was premature and not conclusive is indicated in a later paper by Topley's associate, Lockhart (19), who states that "these experiments (Topley's) were not, however, especially designed to permit the observation of variations in bacterial virulence, other conditions being kept constant, and the conclusions based upon them must, therefore, be regarded as purely inferential and tentative" (page 50).

As, however, Lockhart did not control the host factor in his own tests, and remarks, with reference to his own test inoculations by way of the natural mouth portal of entry, "It is quite certain that, using any number of mice between 20 and 50, the form of the (mortality) curve will vary widely and in a random manner" (page 60), he resorted to intraperitoneal inoculations. This procedure, although useful in some ways, throws no light on the behavior of host and microbe under natural conditions. (Lange's recent experiments also demonstrate this (21).) Four of five of Lockhart's attempts to increase virulence by animal passage were reported as negative; nevertheless he states that while he has "not succeeded in controlling, or varying at will, the virulence of a given strain of *B. aertrycke*" (page 82), he does regard his tests as showing that "fluctuations in the virulence of *B. aertrycke* certainly occur" (page 81).

Another of Topley's collaborators, Wilson (20), later confirmed a view held by us in stating that there is no experimental evidence to support the hypothesis of a geometric rise and fall of bacterial virulence.

In other words, the point of view which has grown out of our own experiments (1, *a*, 1, *e*, 1, *f*, and the present paper) and the latest views of Topley's collaborators are coming more and more nearly to agree with each other.

Turning again to the results reported in the present paper, one may say that when the conditions of host and pathogenic organism are adequately controlled, it is found that mice react to the Friedländer-like bacilli in the same manner as has been stated to occur with other native animal infections,—that is, they do actually differ in respect to their native and acquired ability to resist infection (1, *g*). In the case of the Friedländer-like bacillus infection, as in the instances of other mouse and certain rabbit infectious diseases, we regard the several kinds of clinical manifestations—the septicemic, pulmonary, local or nasal infections, and the carrier state—not as sharply separated clinical conditions, but altogether as the sum total of the disease, each particular manifestation of which representing the momentary degree of resisting power of individual animals affected.

SUMMARY AND CONCLUSIONS.

A spontaneous respiratory disease of mice incited by Friedländer-like bacilli has been described. The bacilli inducing the disease, while morphologically and culturally indistinguishable from the usual varieties of Friedländer bacilli, are antigenically distinct from the common type strains known. The bacilli grow better in cultures at 23°C. than at 37°C.

The disease in mice displayed an incubation period of about 48 hours. About 50 per cent of an exposed population succumbed to septicemic and acute hemorrhagic, pneumonic processes. Among the exposed animals were some individuals which remained apparently well and carried the pathogenic bacilli in their nasal passages.

The several manifestations of the spontaneous disease were reproduced by instilling small numbers of the cultured bacilli into the nasal passages. 48 hours after inoculation, certain mice had already succumbed; the deaths continued to occur, so that by the end of the 2nd week, 70–80 per cent of the animals had died. Among the survivors certain carriers of the bacilli in the nasal passages occurred; a few appeared entirely refractory to the infection. The succumbing

mice showed at autopsy and by culture septicemia and diffuse pneumonic inflammation.

No fluctuation in virulence could be detected in bacilli derived from mice while the disease was spreading spontaneously at the periods of epidemic rise, interepidemic interval, or postepidemic quiescence. Moreover, the bacilli cultured from the nares of apparently healthy carriers were equally pathogenic with those taken from the blood and lungs of animals succumbing quickly.

No rough colony variants were cultured at any phase of the spontaneous disease, although they were readily obtainable by artificial culture. The variant strains proved stable and of low virulence.

This Friedländer bacilli infection in mice takes several clinical courses, depending on variations in host reaction and not depending on bacterial variation. The particular type of infection manifested is determined by the degree of resisting power displayed by the infected animals at the moment that the infection occurred and progressed.

We desire to thank Dr. L. Julianelle for his kindness in titrating the mouse Friedländer strains in his type sera, and Dr. John W. Gowen for analyzing mathematically the experimental data on which a part of this paper is based.

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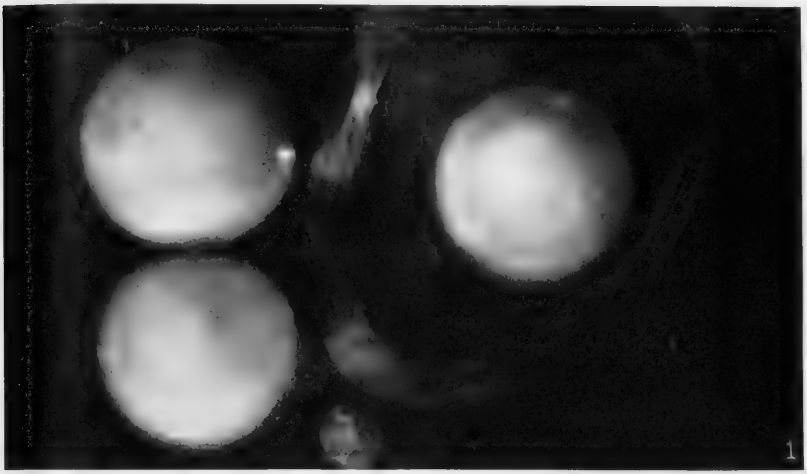
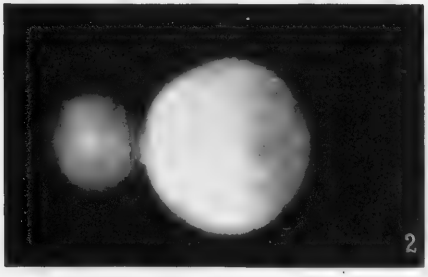
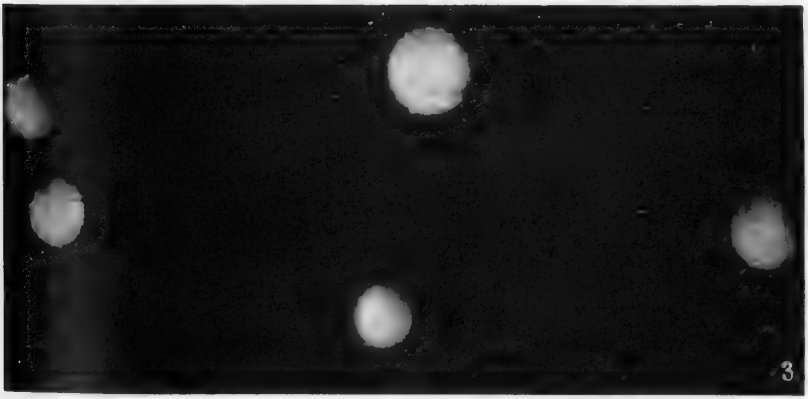
EXPLANATION OF PLATE 33.

FIGS. 1–3. Mouse strains of Friedländer's bacillus. Smooth and rough colonies. × 5.







Photographed by Louis Schmidt.

(Webster: Friedländer bacillus-like infection.)

IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

I. Hemolysin Formation in Normal Rats.

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(Received for publication, February 6, 1928.)

In the course of studies in this laboratory on the effect of suprarenalectomy on antibody formation, it became necessary to determine the hemolysin formation in normal rats. The hemolysin formation in several animals, particularly the rabbit, has been thoroughly investigated, but few studies on hemolysin formation in rats have been reported. In relation to a study of the effect of x-ray on antibody formation, Hektoen (1) produced fairly high hemolysin titers in rats with a small single intraperitoneal injection of sheep cells. Rats present certain peculiarities in immunity phenomena which make such studies of interest. A normal rat produces relatively slight precipitin (2). A state of local hypersensitiveness cannot be induced by injections of a foreign protein (2). Anaphylactic shock is produced with some difficulty (3). Rats withstand huge quantities of various poisons and toxins such as diphtheria (6), tetanus toxin (7) and histamine (4, 5).

Method.

The blood of the sheep was received in a preserving fluid recommended by Rous and Turner (8), consisting of isotonic solutions of glucose and sodium citrate. Cells were never kept longer than 6 days in this fluid and control daily fragility tests were employed. The cells used for injection and testing were washed three times with physiological salt solution to which was added .1 per cent of calcium chloride as recommended by Snapper (9) to prevent a disturbance of the osmotic equilibrium between the red blood cells and the saline solution. Single and multiple injections of varying quantities of antigen were given intraperitoneally and comparative studies of the hemolysin formation were made. Blood was obtained at intervals from the rats by puncturing the heart and withdrawing 1 to 2 cc. of blood. It was necessary to fix the rat on a board specially constructed and to

introduce the needle close to the sternum over the area of cardiac impulse. Repeated punctures at one sitting increase the dangers of fatal hemorrhage, but with practice, fatalities were reduced to less than 5 per cent.

In determining the titer of hemolysin present in the serum, progressive dilutions of the rat serum to be tested were made. .1 cc. of a 5 per cent suspension of sheep cells and 2½ units of complement were added. The total volume of each tube was brought up to 1 cc. with physiological saline. The tubes were incubated at 37° during 30 minutes and readings were made immediately. Traces of hemolysis equivalent to a one plus or lesser reading on a scale in which four plus represents complete hemolysis, were disregarded. That dilution in which hemolysis was partial but definite, that is, a two plus reaction, was read as the titer of the hemolysin present in the serum.

Hemolysin Formation after a Single Intraperitoneal Injection of Sheep Cells.

Twenty adult albino rats raised in the laboratory, from Wistar Institute stock, and all of approximately the same size and age, were used in this series. 1 cc. of a 10 per cent suspension of washed sheep cells was injected intraperitoneally into each rat. The titer was first determined 5 days after the injection and subsequent determinations were made on the 8th, 11th and 14th days. As noted in Table I, the height of the antibody formation is reached on or about the 5th day. The titer then gradually drops and is either very low or disappears entirely 14 days after the injection. The hemolysin titer in normal adult rats injected with 1 cc. of a 10 per cent suspension of sheep cells varies from 1:4000 to 1:24,000. Occasionally a titer of 1:2000 or 1:1600 is noted and in some rats the titer falls below this. The variation is considerable but it is not as striking as in rabbits. Although a high titer is reached with one injection of a small quantity of red blood cells, it is not maintained. The titer drops from 1:16,000 on the 5th day to 1:4000 or 1:2000 on the 8th day, and rapidly to 1:800 or 1:400 on the 11th day, while by the 14th day, it is about 1:200. The average initial titer of the twenty rats was 1:7000 on the 5th day; on the 8th day, the average titer was 1:3270; on the 11th, it was 1:580 and on the 14th day, the average titer was 1:200, although the titer in several animals had practically disappeared at this time.

A single injection of ten times this amount or 1 cc. of undiluted red blood cells was given to eight rats. It is seen from Table II that in these instances no titer exceeded 1:4000 and many titers were much

lower. The average initial titer in the rats in our series was 1:2000 after 5 days as compared with the average titer of 1:7000 of the previous series. The second and third readings were correspondingly lower (see Figs. 1 and 2).

Single injections of .5 cc. of a 10 per cent suspension had the same effect on hemolysin formation as 1 cc. of a 10 per cent suspension.

TABLE I.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
61	6,000	2,000	600	100
62	2,000	200	160	200
63	24,000	8,000	1,600	600
64	2,000	2,000	200	80
65	32,000	16,000	2,000	600
66	3,000	2,000	160	150
27	4,000	Died		
30	300	160	10	20
88	8,000	800	1,000	300
89 A	12,000	1,000	800	100
103 A	1,600	Died		
104 B	400	1,000	80	
105	8,000	2,000	200	
134	1,600	1,600	600	
135	1,000	800	400	200
154	1,000	800	400	160
16	4,000	1,600	600	80
17	12,000	800	600	
23	16,000	2,000		
22	800	160		

However, with injections of .2 cc. of a 10 per cent suspension of sheep cells, antibody titers were considerably less than with 1 cc. of the dilution, although not in mathematical proportion. With single injections of .05 cc. of a 10 per cent suspension or one-twentieth the amount first used, the hemolysin titer rose to a maximum height of 1:800. As will be noted in Table III, the antibody formation in many

TABLE II.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of Undiluted Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
139	4,000	600	400	30
140	4,000	2,000	800	80
141	1,600	400	160	
142	1,600	1,600	800	200
143	800	2,000	2,000	
144	1,600	200	400	
151	1,000	1,000	600	400
152	1,600	1,600		
153	2,000	600	—	400

TABLE III.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of Small Amounts of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8 and 11 Days after the Injection.

Rat No.	Amount injected 10 per cent suspension	Titer		
		5th day	8th day	11th day
	cc.			
90	0.2	400	100	100
94	0.2	2,000	2,000	400
95	0.2	1,600	1,000	800
91	0.1	2,000	2,000	400
96	0.1	1,000	1,000	400
97	0.1	800	1,000	800
92	0.05	200	Died	
93	0.05	800	0	0
98	0.05	1,000	400	Died
136	0.05	400	400	30
137	0.05	400	400	80
138	0.05	200	400	160

instances was approximately one-twentieth as high as with twenty times the quantity of injected antigen. The relative curves with these varying amounts of antigen were similar, the titer disappearing on or about the 14th day (see Fig. 1).

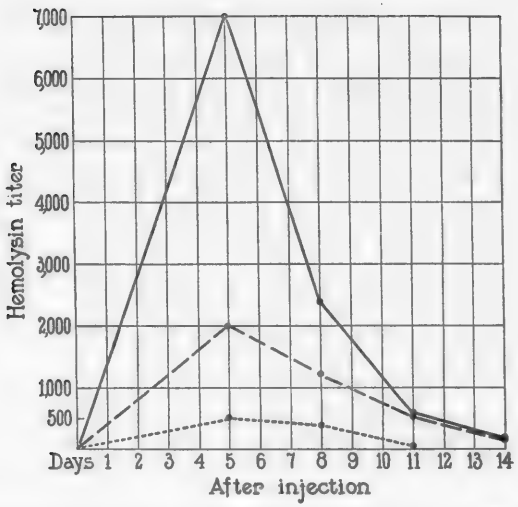

FIG. 1. Curves of average hemolysin titers with varying quantities of antigen.

———— Curve of average hemolysin titer of 20 normal rats injected with 1 cc. of a 10 per cent suspension of sheep cells.

— — — Curve of average hemolysin titer of 9 normal rats injected with 1 cc. of undiluted sheep cells.

--------- Curve of average hemolysin titer of 6 normal rats injected with 0.5 cc. of a 10 per cent suspension of sheep cells.

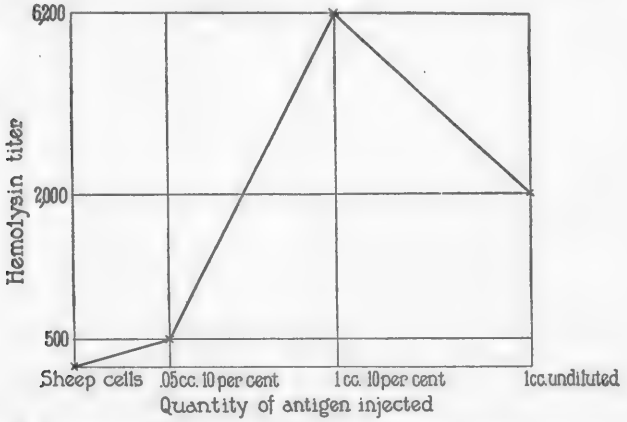

FIG. 2. Antigen-antibody curve in 35 normal rats injected intraperitoneally with varying quantities of sheep cells. The hemolysin titer in each instance is the average optimum titer obtained in each series.

From these experiments, it may be concluded that in normal adult albino rats, relatively high hemolysin titers may be obtained with a single intraperitoneal injection of a small quantity of sheep cells. There is, however, an optimum amount, 1 cc. of a 10 per cent suspension, which produces a definitely high titer. This titer rapidly drops and practically disappears from the blood after a period of 14 to 18 days. Amounts greater than this are not only ineffective in increasing the antibody formation but are followed by lower titers than those obtained with one-tenth the quantity of antigen. Much smaller

TABLE IV.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of Varying Amounts of Washed Sheep Cells at Varying Intervals and Tested 5, 8, 11 and 14 Days after the Last Injection.

Rat No.	No. of injections	Amount of each injection	Interval between injections	Titer			
				5th day	8th day	11th day	14th day
			days				
145	3	1 cc. 1/10	1	2,000	1,600	400	200
146	3	1 " 1/10	1	16,000	1,600	800	400
147	3	1 " 1/10	1	1,000	400	160	100
148	3	1 " undiluted	1	2,000	1,600	400	20
149	3	1 " "	1	1,000	400	30	40
150	3	1 " "	1	600	Died		
18	3	1 " 1/10	5	160	160	300	40
19	3	1 " 1/10	5	100	200	Died	
20	3	1 " 1/10	3	4,000	400	300	200
21	3	1 " 1/10	3	4,000	160	100	100

amounts produce correspondingly lower titers although the ratio is not always mathematically proportional.

Repeated Injections of Red Blood Cells.

Three injections each of 1 cc. of a 10 per cent suspension of sheep cells were given to three rats at daily intervals. The hemolysin titer was tested on the 5th, 8th, 11th and 14th days following the last injection. From Table IV it is seen that the antibody titer tested on the 5th day after the last injection is less than that resulting from a single injection of antigen. The first test was made on the 8th day following the *first* injection of antigen, and if Table IV is compared

with Table I, it is noted that the titer 5 days after the last of three injections repeated at daily intervals with 1 cc. of a 10 per cent suspension is about the same as on the 8th day following a single injection of 1 cc. of a 10 per cent suspension. It would seem that repeated injections at daily intervals failed to increase the antibody formation.

TABLE V.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of a 10 Per Cent Suspension of Washed Sheep Cells at 5 Day Intervals.

Rat No.	No. of injections	Date of injection	Amount injected 10 per cent suspension	Date tested	Titer
			cc.		
1	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	8,000
		9/17/27	0.5	9/17/27	200
				9/22/27	10
				9/26/27	0
2	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	0.5	9/17/27	200
				9/22/27	0
3	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	16,000
		9/17/27	1.0	9/17/27	200
				9/22/27	40
				9/26/27	10
				10/ 1/27	20
4	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	1.0	9/17/27	2,000
				9/19/27	200
				9/22/27	20
				9/26/27	10

*Sera tested before first injection contained no hemolysin.

Three injections at intervals of 3 days, each of 1 cc. of a 10 per cent suspension of red blood cells were given to two rats. The hemolysin titer was determined on the 3rd, 6th, 9th and 12th days following the last injection. Here again, it is noted, from Table IV, that the antibody titer was only slightly less than with a single injection.

Repeated injections at 5 day intervals, each of .5 cc. of a 10 per cent suspension for three injections, were given to several rats. The antibody titer was determined before each injection. From Table V it is seen that in spite of repeated injections of .5 cc. of a 10 per cent suspension of red blood cells, the titer never exceeded the initial rise which it reached 5 days after the first injection. Furthermore, in spite of repeated injections, the titer dropped progressively exactly as in the instances with a single injection. Subsequent injections had practically no effect on the curve of antibody formation produced by the first injection.

Repeated injections of similar amounts of antigen at daily intervals, or at intervals of 3 or 5 days, do not increase the antibody titer nor do repeated subsequent injections of the same amount of antigen modify the curve of hemolysin formation resulting from the first injection.

DISCUSSION.

The curve of the hemolysin titer in healthy rabbits has been carefully studied by Bulloch (10), Sachs (11), Lüdke (12) and Remy (13). Following single or multiple injections of sheep cells, Bessau and Paetsch (14) and Sachs (11) observed a negative phase during which no antibodies were detected in the serum. The length of this negative phase was observed by Sachs to be entirely independent of the amount of blood cells injected, but varied with the method of injection. Following the subcutaneous route, this period was generally 7 days, but in instances in which the intravenous or intraperitoneal route was used, this latent period was reduced to 3 days (Bulloch) or to 2 to 4 days (Sachs). The hemolysin titer then rose to a maximum on the 5th to the 6th day where it was maintained for 24 hours after which it gradually fell. Wolf (15) observed a second rise in rabbits on the 7th to the 10th day. The titer then gradually dropped. There was no direct proportion between the amount of antigen injected and the antibody titer which was produced. Sachs studied the relation of amboceptor to red blood cells and complement in rabbits and found that the appearance of the immune body coincided with the disappearance of the foreign red blood cells from the circulation of the immune animal. Lüdke (16) found that ox cells injected into rabbits subcutaneously completely disappeared in 8 days from the circulation, when hemolysin could first be detected. Probably the immune substances first formed by the injection of the antigen hemolyzed the red blood cells present in the circulation by specific action. In this process complement was also utilized. With the neutralization of the antigen present, the amboceptor became detectable.

In our work we have found that repeated injections of small amounts of red blood cells did not increase the titer of the hemolysin formed by a single injection nor did subsequent injections maintain the titer of hemolysin produced by the first injection. Possibly the amounts injected after the first injection were hemolyzed by existing amboceptor in the serum of the immunized animal and were therefore unable to stimulate further antibody production.

Coca (17) found that after a single injection of 1 cc. of a 10 per cent suspension of sheep cells in rabbits, average titers 1 week after injection reached a maximum of 1:250 (.004). When twenty times this quantity was used, the titer obtained was 1:154 (.0065), definitely lower than with the larger amounts. In rats, we found a similar antibody-antigen relation; with ten times the quantity of antigen, the amboceptor titer dropped. Injections of smaller quantities of antigen in rabbits, Coca found, were more effective in producing a high titer than injections of larger amounts. The observations of Coca and others have shown, however, that in rabbits the number of injections and the intervals between injections influence the hemolysin titer. In rats, the titer following the initial injection of sheep cells was not raised by repeated injections of the same amount of sheep cells. Further subsequent injections had little effect on the curve of the hemolysin formation initiated by the first injection.

SUMMARY AND CONCLUSION.

Hemolysin formation was studied in normal rats. It was found that a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of red blood cells results in an optimum high hemolysin titer 5 days after injection. The titer gradually falls, the hemolysin disappearing from 14 to 18 days after the injection. Larger amounts are less effective in the production of amboceptor. Much smaller amounts produce correspondingly lower hemolysin titers, although the ratio is not mathematically proportional. Repeated injections of 1 cc. of a 10 per cent suspension of red blood cells at daily intervals or at intervals of 3 or 5 days for three injections do not increase the hemolysin titer over that resulting from a single injection. Furthermore, the curve of antibody formation following a single small intraperitoneal injection of red blood cells is not altered by subsequent injections of similar amounts.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

II. Hemolysin Formation in Suprarenalectomized Rats.

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(Received for publication, February 6, 1928.)

There is a great deal of evidence both from morphological and physiological studies that the suprarenal gland plays a significant rôle in the defensive mechanism of the body against intoxication. During the past 7 years numerous observers have established that the resistance of suprarenalectomized rats and rabbits to trauma and to injections of various poisons and toxins is markedly diminished, particularly during the first weeks following the operation. However, only a few studies on the antibody capacity of animals following bilateral suprarenalectomy have been reported. The literature bearing on the relation of the suprarenal gland to the formation of antibodies has been reviewed by Také and Marine (1). Experiments dealing with the antibody capacity of animals following bilateral suprarenalectomy have been limited because of technical difficulties. The most suitable laboratory animals are the rabbit and the rat since a high percentage of these animals survive bilateral suprarenalectomy. Také and Marine found that bilaterally suprarenalectomized rabbits show hemolysin titers distinctly higher than the control animals. Jaffe and Marine (2) observed that rats repeatedly injected with typhoid vaccine 3 weeks following bilateral suprarenalectomy developed agglutinin titers slightly higher than normal control rats. The evidence presented in the literature, however, is of such a contradictory nature that further studies in this field seemed desirable.

The rat was chosen as the experimental animal as over 85 per cent of rats survive bilateral suprarenalectomy in good condition and these present a state of suprarenal insufficiency for a considerable period of

time. The presence of accessory cortical tissue accounts for the recovery and compensation in these animals.

In a preliminary study on the hemolysin formation in normal rats, it was found that high titers were obtained following a single intraperitoneal injection of a small amount of sheep cells (1 cc. of a 10 per cent suspension). This rapid method of immunization, therefore, is definitely advantageous in studying the early period after bilateral suprarenalectomy. The titer was determined at intervals of 5, 8, 11 and 14 days after injection.

Methods.

The methods used have been described in a previous communication (3). Adult albino rats raised in our laboratory, from Wistar Institute stock, were used. The rats were all about 5 months old and of approximately the same weight. The suprarenal glands were removed at a single operation through the posterior route in the manner described by Jaffe (4).* Over 85 per cent of our rats survived 4 weeks or longer after the operation. All rats were carefully autopsied after the experiments and the operated areas were examined, the presence or absence of gross accessory suprarenal tissue determined and the weight of the thymus recorded. Blood for testing was drawn from the heart by puncture.

In the first group of experiments to be reported, we studied the influence of varying the time interval following suprarenalectomy on the titer, keeping the amount of antigen injected constant. In the second group, the quantity of antigen injected was varied, but the time interval kept constant. In the first part of the work, all rats received a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells 2, 7, 14 and 28 days following the operation. A small series of unilaterally nephrectomized rats injected 2 weeks after the operation was tested as a control for operative trauma. In the second group of experiments, two series of suprarenalectomized rats were injected with .05 cc. of a 10 per cent suspension of sheep cells and 1 cc. undiluted sheep cells respectively, 2 weeks after the operation. In preliminary experiments, the range of hemolysin titers in normal rats had been determined with these amounts. Normal rats, and rats in which the perisuprarenal tissue was torn, were tested in both series.

Twenty normal rats were injected with 1 cc. of a 10 per cent suspension of sheep cells and tested 5, 8, 11 and 14 days after the injection. It was found that with this amount, the titer was above 1:2000 in over 70 per cent of the rats. A few rats showed low titers.[1] The average titer of these twenty normals was 1:7000.

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* Ether anesthesia was used in all operative procedures.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 48 Hours after Operation.

Fourteen rats were used in the first series. Of these, six were bilaterally suprarenalectomized, six were normal and two were traumatized by tearing the perisuprarenal tissue. All were injected

TABLE I.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 48 Hours after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
41	Suprarenalectomy	2	1 cc. 1/10	6,000	4,000	800	Dead	160	158	178
42	"	"	"	400	1,600	Dead		150	190	185
43	"	"	"	100	400	80		175	166	326
44	"	"	"	200	400	400	300	165	185	Large
45	"	"	"	16,000	8,000	400	160	200	180	320
46	"	"	"	100	800	20	10	225	220	Small
59	Traumatization	"	"	16,000	10,000	3,000	0?	160	165	162
60	"	"	"	800	800	400	160	150	150	198
61	Control	—	"	6,000	2,000	600	100	183		
62	"	—	"	2,000	600	160	200	193		
63	"	—	"	24,000	8,000	1,600	600	184		
64	"	—	"	2,000	2,000	200	80	170		
65	"	—	"	16,000	32,000	2,000	600	178		
66	"	—	"	3,000	2,000	160	160	185		

intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 48 hours after the operation. Tests were made at intervals of 5, 8, 11 and 14 days. As will be noted in Table I the titers of the suprarenalectomized rats were markedly lower than those of the normal rats. 80 per cent of the suprarenalectomized rats showed an initial titer of 1:400 or less. Two suprarenalectomized rats showed a normal

titer. A comparison between the titer obtained in normal rats[1] and that found in rats following suprarenalectomy reveals a marked depression in hemolysin titer 48 hours after suprarenalectomy. If one examines the curve of antibody formation in those rats showing a low titer, it will be noted that there is a subsequent rise in hemolysin titer at the second reading, the peak of the curve being reached on the 8th instead of the 5th day, as in the normal rats. The depression of antibody formation during the 1st week coincides with the marked depression in resistance of suprarenalectomized rats noted by numerous observers (2, 5–7). It was found by one of us (8, 9) that the height of susceptibility of rats to typhoid and to histamine poisoning is reached during the period from 5 to 7 days after bilateral suprarenalectomy.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 7 Days after Operation.

A second series of thirteen rats, nine of which were suprarenalectomized, two traumatized and two normal, was injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 7 days after the operation. The titers were determined at intervals of 5, 8, 11 and 14 days after the injection. From Table II it is seen that the suprarenalectomized rats still show a definite depression in antibody formation. In two instances where the titer was 1:2000 and 1:3000 a fragment of suprarenal tissue had been left behind. These two instances are therefore excluded from consideration in the series. In this series, 40 per cent gave initial titers below 1:400, 85 per cent below 1:2000 and 14 per cent gave titers of 1:2000 to 1:3000 or within the low titer range of normal rats. The average titer 7 days after the operation was 1:1070 or approximately one-seventh that of normal rats. These figures are slightly higher than those of Series 1, but they still show a definite depression in the hemolysin titer. It is obvious that the suprarenalectomized rats still yield definitely lower antibody titers when the rats were injected with small amounts of sheep cells 7 days as well as 48 hours after the operation. The titers

[1] See Table I of Paper I (3).

of the traumatized animals are likewise depressed. This observation will be discussed elsewhere in this paper.

TABLE II.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 7 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
40	*Incomplete suprarenal-ectomy	7	1 cc. 1/10	2,000	800	10	10	180	195	370
32	Suprarenalectomy	"	"	1,500	1,000	300	200	170	145	375
33	"	"	"	1,600	80	60	Dead	190	180	365
34	"	"	"	900	160	60	40	160	150	298
35	"	"	"	3,000	Dead			160	145	402
13	"	"	"	200	20	20		228		
14	"	"	"	200	100	20		195		
15	"	"	"	100	100	80		200		
38	*Incomplete suprarenal-ectomy	"	"	3,000	800	Dead		275	255	Large
36	Traumatization	"	"	1,800	3,000	200	40	175	175	273
37	"	"	"	800	800	40	80	190	183	223
27	Control	—	"	4,000	Dead			300	300	230
30	"	—	"	300	160	40		250		

*Small fragment of right left *in situ*.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 14 Days after Operation.

A third series of twelve rats, eight of which were suprarenalectomized, two traumatized and two normal, was injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 14 days after the operation and tested 5, 8, 11 and 14 days after the injection. From

Table III it is evident that the antibody titer was higher than in the series injected 7 days after the operation. In only one instance was the initial titer 1:800, and five animals or 60 per cent showed a titer above 1:2000. The average titer was 1:2770 or about one-half the titer of the normal rats. Several of the titers of the operated animals reached the lower normal range. It is interesting that after the 2nd

TABLE III.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 14 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
70	Suprarenalectomy	14	1 cc. 1/10	3,000	Dead			160	150	175
71	"	"	"	1,600	400	300	Dead	168	145	200
72	"	"	"	3,000	Dead			178	178	184
73	"	"			8,000	400		240	160	206
74	"	"	"	1,000	800	800	0	260	260	416
80	*Incomplete suprarenalectomy	"	"	6,000	600	Dead		177	170	
81	Suprarenalectomy	"	"	2,000	1,600	600		161	157	
83	"	"	"	800	1,000	600	600	292	232	
75	Traumatization	"	"	1,000	800	1,600	20	260	270	275
76	"	"	"	160	40	20	0	222	185	295
88	Control	—	"	8,000	800	800	300	148	145	275
89 A	"	—	"	12,000	1,000	800	100	200	220	295

*Small fragment left *in situ*.

week, the capacity to form antibodies in response to injections of small amounts of sheep cells definitely rises, but is still somewhat below that of normal rats. As has been established by Jaffe (4) and Marine, there is a marked hyperplasia of the thymus and the lymph nodes 2 weeks after suprarenalectomy. This may be a factor in the explanation of the compensatory rise in antibody formation at this time.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 4 Weeks after Operation.

A fourth series of nine rats, four of which were suprarenalectomized, two traumatized and three normal, was injected with 1 cc. of a 10 per cent suspension of sheep cells 4 weeks after operation. Two suprarenalectomized rats gave titers below 1:1000. At 4 weeks, the titer of hemolysin formation had not returned to normal but was definitely lowered. No definite comparison can be made from this small series.

TABLE IV.

Hemolysin Formation in Bilaterally Suprarenalectomied Rats, Intraperitoneally Injected 28 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8 and 11 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer			Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day			
		days					*gm.*	*gm.*	*mg.*
51	Suprarenalectomy	28	1 cc. 1/10	2,000	4,000	Dead	175	160	
52	"	"	"	200	Dead		175	170	552
53	"	"	"	300	200	160	175	145	
55	"	"	"	1,000	Dead		140	130	313
57	Traumatization	"	"	160	200	80	220	280	185
58	"	"	"	3,000	800	160	170	—	—
103 A	Control	—	"	1,600	Dead		250	260	223
104 B	"	—	"	400	1,000	80	245	250	186
105 C	"	—	"	8,000	2,000	200	290	300	261

From these four series it may be concluded that in biliaterally suprarenalectomized rats, the hemolysin formation resulting from a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells is depressed in all periods up to and including 4 weeks following the operation. The depression is greatest during the 1st week, lessens slightly during the 2nd week and diminishes considerably during the 3rd week. After 4 weeks, the hemolysin titer is still below the normal reading.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of .05 Cc. of a 10 per Cent Suspension of Sheep Cells 2 Weeks after Operation.

The second group of experiments was carried out to determine the effect of varying amounts of antigen on the antibody formation.

TABLE V.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 14 Days after the Operation with .05 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8 and 11 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer			Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day			
		days					*gm.*	*gm.*	*mg.*
103	Suprarenalectomy	14	0.05 cc. 1/10	300	300	40	180	170	175
107	"	"	"	300	200	20	195	180	200
108	"	"	"	200	300	200	140	110	160
109	"	"	"	200	200	40	200	185	310
110	"	"	"	400	200	10	190	170	271
111	"	"	"	40	40	Dead	260		
112	Traumatization	"	"	200	80	"	275	300	176
113	"	"	"	40	0	"	300	305	196
114	"	"	"	20	20	10	260	260	140
136	Control	—	"	400	400	30	184		
137	"	—	"	400	400	20	225		
138	"	—	"	200	400	160	166		
92	"	—	"	200					
93	"	—	"	800	0	0			
98	"	—	"	1,000	400		155	155	

In the first series of this group, fifteen rats were used, six of which were suprarenalectomized, six normal and three traumatized by tearing the perisuprarenal tissue. All were injected 2 weeks after the operation intraperitoneally with .05 cc. of a 10 per cent suspension of sheep cells (brought up to a volume of .5 cc.). This quantity of antigen represents one-twentieth the amount used for injection

throughout the first group of experiments. It will be noted from Table V that the range of hemolysin titers in normal rats was from 1:200 to 1:1000, the average titer being 1:500. In the suprarenalectomized group, the titers ranged from 1:20 to 1:400, the average titer being 1:240 or one-half the normal. Here as in the first group of experiments, the hemolysin formation in rats injected 2 weeks after bilateral suprarenalectomy is less than that of the control rats. It is significant that as in the first group, the traumatized controls gave lower titers than either the normal or suprarenalectomized rats of this period.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of Undiluted Sheep Cells 2 Weeks after Operation.

In the second series of the second group eighteen rats were used, six of which were bilaterally suprarenalectomized, nine normal and three traumatized in the perisuprarenal area. All were injected 2 weeks after the operation with 1 cc. of undiluted sheep cells. In the normal rats of this series, hemolysin titers in no instance exceeded 1:4000. 65 per cent showed titers below 1:2000. These titers are strikingly lower than those found in the normal rats injected with 1 cc. of a 10 per cent suspension (one-tenth the amount).[1] The average titer in the series of rats injected with 1 cc. of undiluted sheep cells was 1:2000 as compared with 1:7000, the average titer in the normal rats receiving one-tenth the amount of antigen.

Coca (10) found that small quantities of sheep cells are more effective in the production of hemolysin in rabbits than larger amounts. There is a definite optimum antigen amount for the production of hemolysin in rats. Larger or smaller amounts are less effective.

In contrast with this drop in hemolysin titer in *normal rats* injected with 1 cc. of undiluted sheep cells, the *suprarenalectomized* series injected 2 weeks after the operation with the same amount of antigen shows a definitely higher titer than that given by the normal rats. 100 per cent gave a titer of 1:2000 or above, 90 per cent gave a titer above 1:4000 and 50 per cent, a titer above 1:8000! In the normal rats, only 40 per cent gave titers above 1:2000 and none gave titers above 1:4000. 60 per cent gave titers of 1:1600 or less. The average

titer in the suprarenalectomized animals injected with large amounts of antigen was 1:7330. Quantities of 1 cc. of a 10 per cent suspension produced slightly lower titers in suprarenalectomized rats when injected 2 weeks after the operation than in normal rats. Quantities

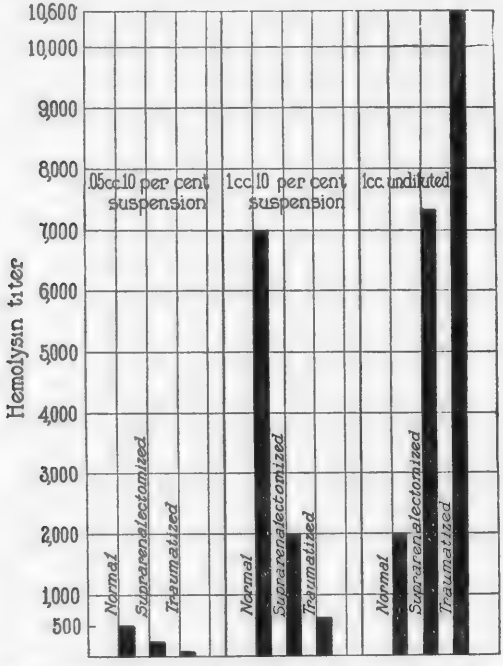

FIG. 1. The relation of hemolysin formation to the quantity of antigen injected in normal rats, in suprarenalectomized rats and in rats in which the perisuprarenal tissue had been traumatized. All rats were injected intraperitoneally 2 weeks after the operation and tested 5 days after the injection. The hemolysin titer in each group is the average optimum titer obtained in the series of rats with .05 cc. of a 10 per cent suspension, 1 cc. of a 10 per cent suspension and 1 cc. of undiluted sheep cells, respectively.

ten times this amount, however, produced titers definitely higher in the suprarenalectomized rats than in the normal rats. The optimum antigen amount necessary to produce the highest titer in suprarenalectomized rats injected 2 weeks after the operation, therefore, is

evidently ten times the optimum necessary to produce the highest titer in normal rats.

The difference in curves illustrating the antigen-antibody ratio can be readily observed in Fig. 1. The titers obtained in suprarenalecto-

TABLE VI.

Hemolysin Formation in Bilaterally Suprarenalectomied Rats, Intraperitoneally Injected 14 Days after the Operation with 1 Cc. of Undiluted Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
115	Suprarenalectomy	14	1 cc. undiluted	6,000	1,000	Dead		250	195	313
116	"	"	"	16,000	3,000	1,800	200	250	210	—
117	"	"	"	8,000	4,000	2,000	200	200	180	214
118	"	"	"	8,000	3,000	Dead		230	215	516
119	"	"	"	2,000	800	400		265	210	375
121	"	"	"	4,000	3,000	1,000		270	250	320
122	Traumatization	"	"	8,000	2,000	800	160	270	300	210
123	"	"	"	18,000	4,000	Dead		168	160	155
124	"	"	"	6,000	3,000	160		225	220	410
139	Control	—	"	4,000	600	400	30			
140	"	—	"	4,000	2,000	800	80			
141	"	—	"	1,600	400	160				
142	"	—	"	1,600	1,600	800	200			
143	"	—	"	800	2,000				200	170
144	"	—	"	1,600	200	200			200	110
151	"	—	"	1,000	1,000	1,000	400			
152	"	—	"	1,600	1,600					
153	"	—	"	2,000	600	400	200		505	115

mized rats injected with 1 cc. undiluted sheep cells correspond to the titers obtained in normal rats injected with one-tenth this quantity, but exceed titers obtained in normal rats injected with the same quantity.

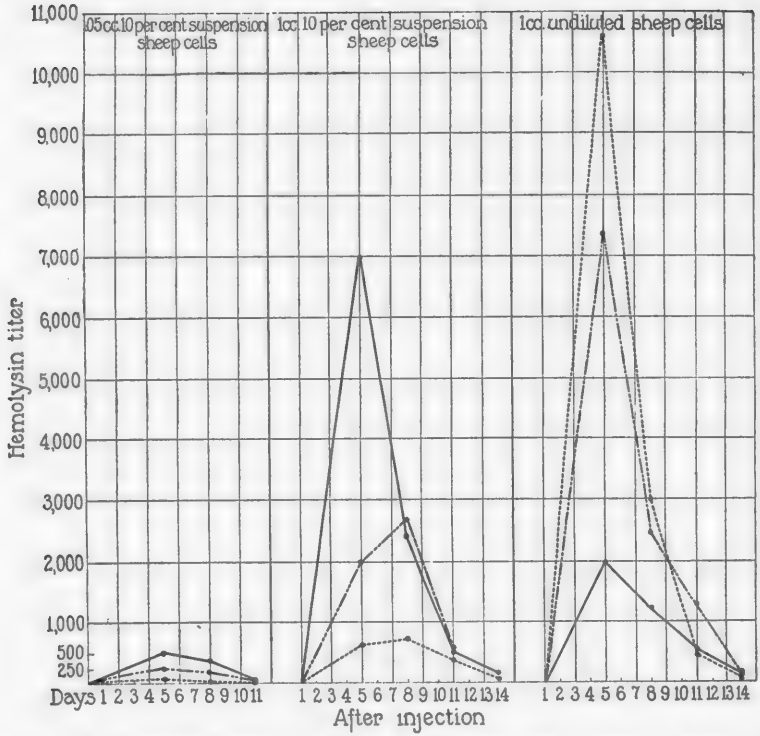

FIG. 2. Hemolysin formation with varying quantities of antigen in normal, suprarenalectomized and traumatized rats. The curves indicate the course of the average hemolysin formation following a single intraperitoneal injection of .05 cc. of a 10 per cent suspension, 1 cc. of a 10 per cent suspension and 1 cc. of undiluted sheep cells respectively, 2 weeks after the operation.

——— Normal rats.

— — — — Suprarenalectomized rats.

--------- Traumatized rats.

Hemolysin Formation in Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 2 Weeks after Traumatization of the Perisuprarenal Tissue.

Throughout the experiments reported in this paper, it had been observed that the traumatized control rats in all postoperative periods

TABLE VII.

Hemolysin Formation in Bilaterally Traumatized Rats, Intraperitoneally Injected at Varying Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after the Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
59	Traumatization	2	1 cc. 1/10	16,000	20,000	3,000		160	165	162
60	"	"	"	800	800	400	160	150	155	198
36	"	7	"	1,800	3,000	800	40	175	175	273
37	"	"	"	800	800	40	80	190	183	223
75	"	14	"	1,000	800	1,600	80	260	270	275
76	"	"	"	160	40	20	0	222	185	295
57	"	28	"	180	200	80		220	280	185
58	"	"	"	3,000	800	160		170	280	185
125	"	14	"	20	40	40	100	210	280	190
127	"	"	"	800	160	100	150	205	265	171
128	"	"	"	20	200	20	100	216	280	155
129	"	"	"	800	600	200		235	250	
130	"	"	"	400	800			210	280	140
131	"	"	"	800	1,600	200	100	165	185	75
132	"	"	"	300	1,600	400	100	190	210	80
133	"	"	"	2,000	1,600	400	100	155	195	95

including 4 weeks gave, with few exceptions, strikingly low titers. This finding was so striking that in a series of rats the perisuprarenal tissue was traumatized, and the rats were subsequently injected with 1 cc. of a 10 per cent suspension of sheep cells 2 weeks after the operation. The titers were tested on the 5th, 8th, 11th and 14th days after injection. Rats 125 to 133 tabulated in Table VII record the results

of this series. One of the nine rats gave a titer of 1:2000; all the others were below 1:800. These titers corresponded in general with those given by the suprarenalectomized rats during the 1st week. This profound depression was still observed in Rat 57, 4 weeks after the operation.

In four rats, one kidney was removed as an operative control. These rats were injected 2 weeks after the operation with 1 cc. of a 10 per cent suspension of sheep cells. As indicated in Table VIII the titers were the same as in the normal unoperated rats.

TABLE VIII.

Hemolysin Formation in Unilaterally Nephrectomized Rats, Intraperitoneally Injected 14 Days after Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after the Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						*gm.*	*gm.*	*mg.*
99	R. removed	14	1 cc. 1/10	8,000	400	400		140	145	110
100	"	"	"	12,000	200	600		145	145	233
101	"	"	"	1,600	200	200	80	230	235	230
102	"	"	"	32,000				200	205	209

It is justifiable to conclude from these observations that traumatization of the perisuprarenal tissue in some way results in a prolonged depression in antibody-forming capacity. This depression persists at the same low level from a few days after the operation to at least the 5th week. Traumatization of the perisuprarenal tissue in rats results in a depression of hemolysin formation which is even more marked than in a corresponding suprarenalectomized series at the same postoperative interval. It is suggested that traumatization of the perisuprarenal area probably results in severing or injuring the sympathetic or parasympathetic nerves innervating the suprarenals. This injury may cause a depression of function of cortex as well as medulla.

It has been thoroughly established by the work of Dreyer (11), Tscheboksaroff (12), Stewart and Rogoff (13) and others that the output of epinephrine from the suprarenal gland is controlled by a nervous mechanism and that interruption of the nervous pathway results in a diminution of the epinephrine output. The extent of the nervous control of the cortex, however, is entirely unknown. Depression of antibody-forming capacity to small amounts of antigen may be an expression of impaired suprarenal function. With the removal of suprarenal glands in rats, regeneration of thymus and lymphoid tissue occurs. This hyperplasia does not occur following traumatization of perisuprarenal tissue. This lymphoid hyperplasia may be in some way responsible for the partial recovery in suprarenalectomized rats of the antibody-forming capacity 2 weeks after the operation.

DISCUSSION.

It has been thoroughly established that the resistance of suprarenalectomized rats and rabbits to injections of various toxins is markedly diminished particularly during the first few weeks following the operation. As has been pointed out by Marine (14) and others, it is not established that antibody formation is necessarily an indication of resistance. However, it would appear *a priori* that during the height of diminished resistance, some depression in antibody reaction capacity may be expected. Our studies indicate that certainly in the 1st week following suprarenalectomy, a marked hemolysin depression does occur and is most marked at the height of susceptibility. The significant chemical changes in the blood plasma known to follow suprarenalectomy may play a rôle in this depression. Physicochemical changes such as change in the dispersion of the proteins in the serum, slight variations in the pH, variations in the concentration of metallic ions in the serum may all play a part in the depression in hemolysin during the early period following suprarenalectomy. However, the serum of suprarenalectomized rats when added in varying concentrations to sera of known hemolytic titer did not produce any depression of hemolysis. This adds further evidence that the depression in antibody formation in the early period after suprarenalectomy may be due to a disturbance in the antibody-forming mechanism of the body. This depression coincides with the di-

minished resistance of rats to toxins and poisons during the first 2 weeks and may be intimately related to it.

Marine and his coworkers (1, 4) found an increase in antibody formation following suprarenalectomy. They employed sublethal amounts of antigen. Their results are comparable with those in our work in which large amounts of antigen were used. Periods earlier than 3 weeks were not tested by these investigators. The effects of comparably small amounts of sheep cells were not tried. The rise in antibody production following the use of large amounts of sheep cells 2 weeks after suprarenalectomy may be indirectly dependent on loss of suprarenal function and on the consequent lymphoid hyperplasia following suprarenalectomy.

Traumatization of the perisuprarenal tissue had an effect similar to that of suprarenalectomy in depressing hemolysin formation particularly during the 1st week following the operation. This depression persisted in all periods up to 4 weeks. It is interesting to note, however, that with ten times the normal optimum antigen amount, the titers in the traumatized as well as in the suprarenalectomized rats were higher than in normal rats.

SUMMARY.

In a large series of suprarenalectomized rats, hemolysin formation, to a fixed amount of sheep cells injected intraperitoneally (1 cc. of a 10 per cent suspension), was studied 48 hours, 7, 14 and 28 days after operation. The hemolysin formation was studied in suprarenalectomized rats injected 2 weeks following the operation, with one-twentieth and ten times this amount. Hemolysin formation in rats traumatized by tearing the perisuprarenal tissue and injected 2 weeks after the operation with 1 cc. of a 10 per cent suspension of sheep cells was studied and the results compared with those in the normal control rats. The effect of the operative procedure was controlled by removing one kidney in a series of rats and determining the antibody response to sheep cells.

1. Bilateral suprarenalectomy in rats subsequently injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells resulted in a depression of hemolysin titer during 5 weeks following the operation, the depression being most marked during the 1st week.

2. Bilaterally suprarenalectomized rats injected intraperitoneally 2 weeks after operation with 1 cc. of undiluted sheep cells gave hemolysin titers higher than did normal rats.

3. The quantity of antigen necessary to yield the maximum titer in suprarenalectomized rats 2 weeks after operation is ten times the quantity necessary to yield the same titer in normal rats.

4. Traumatization of the perisuprarenal tissue in rats produced the same effect on the antibody-forming capacity as suprarenalectomy.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

V. The Elevation of Temperature in the Pneumonic Lung.

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Plates 34 and 35.

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INTRODUCTION.

Two of us (C. B. and R. C.) have shown that the systemic temperature can be raised by diathermy, but that there is relatively little local heat developed in the normal lung (1). Thermocouples placed in the lobes of the lungs of anesthetized dogs seldom registered more than 0.4°C. higher than simultaneously recorded rectal temperatures. This is true in spite of the fact, now established (2), that the high frequency current produced by a diathermy machine actually penetrates the body and passes through the lungs, generating heat in the tissues it traverses. Why there is no marked local elevation of temperature in these tissues has been shown to be due to the fact that the heat generated is rapidly disseminated throughout the body by the circulating blood. The blood leaving the lungs can be shown to have been heated by the current (3). If, however, the pulmonary circulation to one lung is interrupted, a precipitous rise in temperature occurs in the ischemic lung (4). The degree of local heating which occurs under these circumstances will depend upon the extent of interference with the pulmonary circulation. This can be shown by interrupting the flow of blood through one branch of the pulmonary artery, leaving the veins patent and the bronchial circulation intact. There results from this procedure an increase of temperature in the ligated lung amounting to about 1.5°C. in excess of the temperature in the normal lung. The increase occurs immediately after the artery is clamped, while the subsequent rate of heating is similar to that in the control

lung. When, on the other hand, the veins to one lung are ligated, the local heating is far greater than under the conditions just described. Reference to the figures in Paper II of this series will bring out these facts.

From the foregoing, the implication seems obvious that a disease process accompanied by impairment of local circulation may be expected to provide conditions consistent with the production of local heat by the passage of high frequency currents through the region involved. That the pulmonary consolidation of pneumonia represents a disease process in which local circulatory impairment exists, there is evidence to believe. We cite in this connection the pathological studies of Kline and Winternitz (5), the injection preparations of Gross (6) and the inferences to be derived from analyses of the oxygen saturation of the arterial blood (7). The present study was undertaken for the purpose of discovering whether the consolidated lobe could in fact be heated above the temperature of the uninvolved, relatively normal lung tissue. The difficulties attending the production of experimental lobar pneumonia in laboratory animals are well known. Though the pathological lesions which presented themselves in the experiments about to be reported were not perhaps identical with those commonly seen in human lobar pneumonia, they, at least, may be said to simulate this condition and to represent a more or less complete consolidation of a lobe, or the major part of it.

We have found that such a consolidated lobe can be heated by a diathermy current of the strength generally used in therapy to a point approximately 1°C. to 2°C. above the temperature of the surrounding normal lobes.

Methods.

Dogs were used in all of these experiments. During deep ether anesthesia they were inoculated by the method of intrabronchial insufflation described by Lamar and Meltzer (8). Actively growing broth cultures of two varieties of organisms were used: *B. friedlænderi* Type B and Pneumococcus Type I. An effort to maintain virulence was made in the case of Friedländer's bacillus by passage through guinea pigs, and in the case of pneumococcus by mouse passage. The cultures were injected intrabronchially in volumes varying from 1 to 3 cc. per kilo body weight. Even with careful control of conditions the results of these inoculations were uncertain and variable. Some animals died within 24 hours of inoculation, before the appearance of a pulmonary lesion. Others survived ap-

parently unharmed. It was necessary to select animals for use on the basis of x-ray evidence of pneumonic consolidation.

The method of lung temperature measurement and diathermy administration was the same as that previously described (1). Observations were made on the dogs after they had been anesthetized by the intravenous injection of a 10 per cent solution of barbital-sodium, the dose not exceeding 0.3 gm. per kilo body weight. The low initial temperatures often observed may be ascribed in part to the result of infection, in part to heat loss resulting from anesthesia. This was difficult to prevent, since we thought it best to use no hot pad or source of heat other than diathermy. Considerable practice was necessary before we learned to lodge the thermocouple needle in the substance of the consolidated lobe. Owing to the differences in the shapes and sizes of the dogs it was difficult to find any external landmarks which would act as guides for the insertion of the needle into any particular area. In the majority of instances the lower lobe alone was consolidated. In order to get a thermocouple into it the needle was usually inserted close to the spinal column, through the 9th or 10th intercostal space. Even with this guide the element of luck remained a large one. Other thermocouples were inserted in symmetrical positions into the opposite healthy lobes. At the close of each experiment an autopsy was done in which the positions of all thermocouples were located. The gross appearance of the lungs was described in relation to the site of the thermocouples and the lungs were usually saved either for injection preparations or for microscopic examination.

EXPERIMENTAL.

Experiment D 36.—May 19, 1927. At 2:45 p.m. a male mongrel fox terrier weighing 12.7 kilos was given ¼ gr. morphine sulfate subcutaneously. About 15 minutes later it was etherized deeply. A short piece of rubber tubing was passed through the larynx into the trachea, and through this was inserted a soft rubber tube 30 cm. long, with an outside diameter of 3 mm. When the bronchial catheter was in place its proximal end was connected with the nozzle of a record syringe previously filled with 26 cc. of a 4 hour broth culture of *B. friedlænderi*. The whole mass of culture was then injected and forced into the lung with a small quantity of air. There was no cough following injection. The animal made a quick recovery from the anesthetic.

May 20, 12, noon. The dog looked moderately sick. The respirations were rapid, but not labored. Rectal temperature was 39.5°C.

May 21, 9 a.m. The dog was now definitely sick. An x-ray photograph showed a slight shadow at the base of the left lung (Fig. 1).

May 21, 10 a.m. The animal was anesthetized by the intravenous injection of 27 cc. of a 10 per cent solution of barbital-sodium.

10:30 a.m. The sides of the dog's thorax were shaved and lead-tin electrodes 3 × 4 inches square were applied to the shaved skin. To assure perfect contact several layers of gauze moistened with a solution made from mixing equal parts of

glycerol and saturated saline were interposed between skin and electrodes. A rectal thermometer was put in place and the thermocouples were thrust through the chest wall into the lungs.

11:10 a.m. The larynx was intubated to facilitate breathing.

11:14 a.m. to 1:50 p.m. Thermocouple readings were made at 10 to 15 minute intervals (Table I). The diathermy current was turned on at 11:29 a.m. and ran continuously until 1:50 p.m.

1:55 p.m. The dog was killed by the intravenous injection of 20 cc. of a saturated solution of $MgSO_4$, the trachea being clamped in inspiration.

The thorax was opened and the position of the thermocouples and condition of the lungs established.

Thermocouple 1 was buried in the substance of the lower portion of the left upper lobe, near the lung root. It was lying in a discolored, congested area of the lobe which was, however, air-containing.

Thermocouple 3 had transfixed the upper pole of the left lower lobe and lay in that portion of the ventral lobe which adjoins the hilum. This part of the lobe was dark and definitely abnormal in appearance, as was the left lower lobe immediately adjoining it, which was purplish, boggy and non-air-containing. No thermocouple was found in this left lower lobe.

Thermocouple 2 was found in the substance of the right upper lobe in what was apparently normal lung tissue.

Thermocouple 4 lay in the pleural cavity projecting into the interlobar space and surrounded by healthy tissue.

To summarize the situation found at autopsy: Two of the thermocouples, Nos. 1 and 3, were found within the substance of the left lung, which was the seat of a pneumonic process. Although lying in pathological tissue, neither had penetrated the lower lobe, where the consolidation was most pronounced. The other two thermocouples, Nos. 2 and 4, lay in, or in close proximity to, the normal tissue of the right lung.

As to the temperature changes recorded by these thermocouples: Both thermocouples, Nos. 1 and 3, showed throughout the duration of current flow a greater rate of heating than Nos. 2 and 4, which were lodged in the opposite normal lung. The rate was greater in No. 3 than in No. 1, corresponding in this to the degree of structural damage found at the respective sites of these thermocouples. Comparing the temperatures developed in the left lung and right lung (Table I, Text-fig. 1) one sees, for example, in Thermocouple 2 (right healthy lung) a rise of 4.52°C. in 2 hours and 21 minutes of diathermy. The rectal temperature during this period had risen 4.42°C., while Thermo-

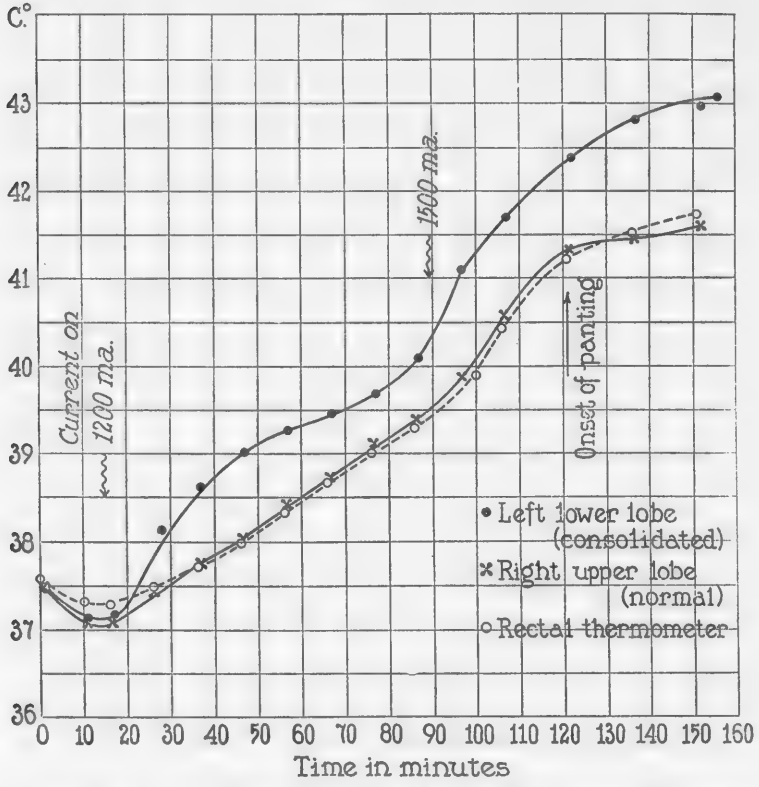
TABLE I.

Temperature Changes Recorded in Experiment D 36.

Time	Current	Rectal thermometer	Thermocouple 1 in left lung (pathological)	Thermocouple 2 in right lung (normal)	Thermocouple 3 in left lung (pathological)	Thermocouple 4 in right lung (normal)	Thermocouple 5 in subcutaneous tissue under left electrode	Thermocouple 6 on skin under left electrode	Thermocouple 7 on skin under right electrode	Pulse rate per min.	Respiratory rate per min.	Remarks
	ma.	°C.										
11:14	0	37.57*	37.50	37.48	37.48	37.51	35.98	35.17	36.07	29	166	Respirations irregular
11:24	0	37.31	37.19	37.08	37.14	37.16	35.98	35.07	36.00	37	168	
11:30	1200	37.28	37.33	37.09	37.68	37.20	47.78	38.12	39.15	38	168	Current on at 11:29
11:40	1200	37.48	37.62	37.42	38.13	37.43	50.13	41.40	41.30	28	164	
11:50	1200	37.71	37.94	37.75	38.62	37.74	52.11	43.31	42.71	31	166	
12:00	1200	37.98	38.26	38.01	39.01	38.05	51.87	43.88	42.86	30	164	
12:10	1200	38.32	38.68	38.41	39.26	38.43	50.68	42.85	42.48	24	170	
12:20	1200	38.66	38.92	38.72	39.46	38.75	50.77	42.88	42.45	23	172	
12:30	1200	39.00	39.18	39.12	39.69	39.16	50.52	42.98	42.45	23	180	
12:40	1500	39.29	39.52	39.39	40.10	39.41	50.49	43.16	42.55	23	184	Current increased to 1500 ma. at 12:43½
12:50	1500	39.89	40.14	39.88	41.09	39.99	56.30	45.29	44.38	25	186	Rectal temperature read at 12:54
1:00	1500	40.43	40.84	40.57	41.69	40.68	55.72	45.92	44.81	24	188	
1:15	1500	41.21	41.70	41.32	42.38	41.46	55.00	45.92	45.05		190	Panting began suddenly at 1:16
1:30	1500	41.52	42.07	41.45	42.82	41.60	54.65	45.88	45.32		194	Panting.
1:45	1500	41.73	42.25	41.60	42.98	41.75	54.12	45.88	45.29		204	Panting.
1:50	1500				43.08							Current off at 1:50½
1:50¼	0				42.51							
1:50½	0				42.32							

*The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

couple 3, in diseased lung, showed an increase of 5.84°C., or 1.32°C. above the temperature developed in the control lung. Such an increase corresponds in order of magnitude to the change shown to occur



TEXT-FIG. 1. Experiment D 36. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature. After 107 minutes of current flow the dog suddenly began to pant. This resulted in cooling of the normal lung, which thereupon fell below the rectal temperature. No such change occurred in the pathological lobe.

during diathermy in a lung after clamping its pulmonary artery (4). The experiment just described (D 36) was repeated in six other dogs. Because of the inconstant pathological changes produced by Fried-

länder bacillus insufflations, and because of the inherent uncertainty of directing the thermocouple needles into both consolidated and healthy lung tissue, it was impossible to demonstrate heating of the pathological lobe in all experiments. We have deliberately chosen for publi-

TABLE II.

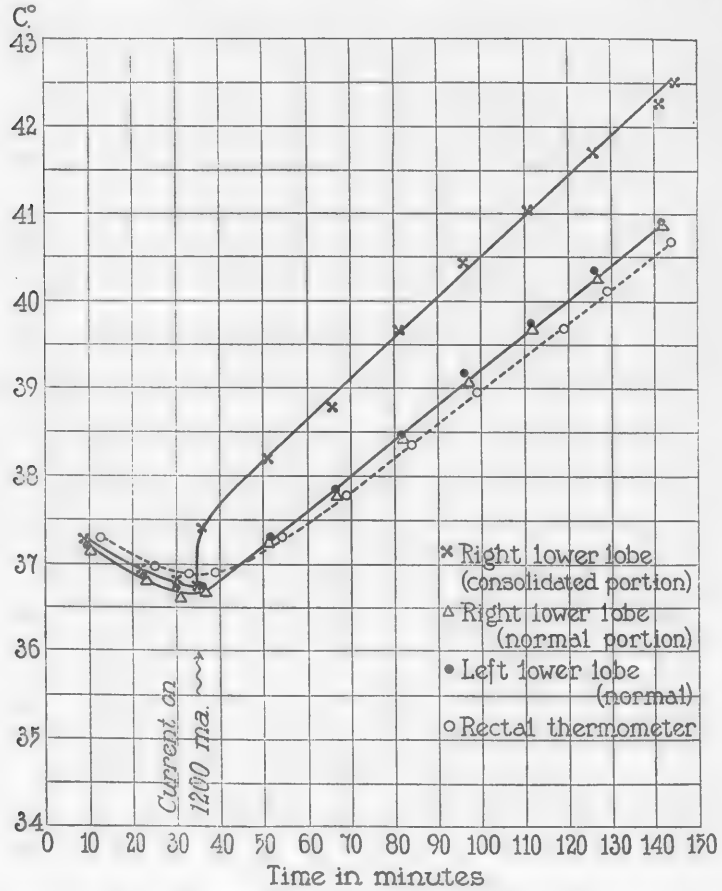
Temperature Changes Recorded in Experiment D 38.

Time	Current	Rectal thermometer	Thermocouple 1 in right lung (consolidated)	Thermocouple 2 in left lung (normal)	Thermocouple 3 in right lung (pathological)	Thermocouple 4 in left lung (normal)	Thermocouple 5 in right lung (pathological)	Thermocouple 6 on skin under right electrode	Thermocouple 7 on skin under left electrode	Pulse rate per min.	Respiratory rate per min.
	ma.	°C.									
11:39	0	37.30*	37.29	37.20	37.18	37.18	37.29	34.52	34.76		48
11:52	0	36.98	36.94	36.86	36.82	36.84	36.92	34.60	34.76	184	52
12:00	0	36.89	36.82	36.73	36.62	36.68	36.77	34.80	34.80		64
	On										
12:05	1200		36.75								
12:06	1200	36.90	37.42	36.75	36.68	36.82	36.90	37.13	38.11	208	64
12:21	1200	37.31	38.20	37.31	37.25	37.44	37.46	40.78	41.02	200	62
12:36	1200	37.79	38.77	37.85	37.79	37.96	38.07	42.90	42.68	200	56
12:51	1200	38.37	39.67	38.49	38.44	38.60	38.70	43.59	43.14	200	50
1:06	1200	38.96	40.43	39.18	39.07	39.28	39.35	43.71	43.38	196	46
1:21	1200	39.07	41.01	39.77	39.67	39.90	39.98	43.75	43.83	196	44
1:36	1200	40.12	41.70	40.37	40.27	40.50	40.61	44.06	44.29	196	46
1:51	1200	40.69	42.25	40.90	40.86	41.10	41.26	44.73	44.81		
	Off										
1:55			42.51								
1:55¼	0		41.89								
1:55½	0		41.77								
1:55¾	0		41.70								
1:56½	0			41.02							

* The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

cation in detail those experiments in which a certain degree of local heating was produced. It should be emphasized that failure to find local heating could always be accounted for on the basis of no consolidation or malposition of thermocouples, except in one instance, Experiment D 49, to be referred to below.

Experiment D 38.—On May 25, 1927, at 2:45 p.m., a male mongrel weighing 12.6 kilos, after a preliminary subcutaneous injection of ¼ gr. morphine sulfate, was given, under deep ether anesthesia, an intrabronchial insufflation of 25 cc.



TEXT-FIG. 2. Experiment D 38. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

of a 4¾ hour culture of *B. friedlænderi*. The next afternoon at 4:20 the dog looked moderately sick and showed a rectal temperature of 40.1°C. An x-ray photograph showed a slight shadow at the base of the right lung (Fig. 2). On

May 27, at 9:30 a.m., the dog was coughing occasionally, but looked less sick than on the previous day. The animal received an intravenous injection of 37 cc. of a 10 per cent solution of barbital-sodium. When a satisfactory state of anesthesia had been achieved, electrodes were applied and thermocouples inserted in the usual manner. A current of 1200 milliamperes was passed through the thorax for 1 hour and 50 minutes. Immediately after the current was turned on it was found that Thermocouple 1 which had been directed into the right lower lobe through the anterior chest wall was recording a more rapid rise in temperature than any of the others within the thorax or than the rectal thermometer. This thermocouple, No. 1, was found at autopsy to be buried in the right lower lobe which had the appearance of liver, except for an air-containing portion on its anterior aspect. Both of the other thermocouples in the right lung were found in the upper portion of the lower lobe where the consolidation was less marked. The lesion on the right side increased in severity from above downward, which may perhaps account for the fact that the final temperatures observed in the right lower lobe showed a gradation from above downward: 40.86°C., 41.26°C., 42.25°C. The final temperature recorded by this thermocouple represented a rise of 5.45°C. in 1 hour and 50 minutes (Table II, Text-fig. 2), as compared with a rise of 4.17°C. and 4.42°C. respectively in thermocouples, Nos. 2 and 4, located in the normal left lower lobe. The increase in rectal temperature during the same interval amounted to 3.80°C. The final temperature of the skin under the electrodes was 44.73°C. on the right side, 44.81°C. on the left. The surface temperature cannot, therefore, be held responsible for the observed difference in internal temperature.

The findings in Experiment D 38 are quite comparable to those in D 36. In interpreting them the many controls supplied by our previous studies should be borne in mind, where it was clearly shown that the normal lung with intact circulation could be heated to an average maximum of 0.35°C. above the rectal temperature. In only one experiment of 13 did the temperature in the lung mount as high as 0.6°C. above the rectal during the course of diathermy. Since this animal died unaccountably while the experiment was in progress it is probable that the slight increase in heating can be explained on the basis of a failing pulmonary circulation.

Experiment D 48.—The procedure outlined in the previous experiments was again carried out in a female mongrel weighing 8.8 kilos. While etherized it received, on Nov. 6, 1927, 10 cc. of a *B. friedlænderi* culture intrabronchially. The next afternoon lung temperature measurements were recorded during diathermy. Autopsy showed the left lower lobe to be hepatized, while the upper and ventral lobes were air-containing and normal in appearance. The right lung was normal except for congestion and nodular consolidations along the posterior

margin of the lower lobe, most marked near the hilum. The caudal lobe, as well, was congested. The most marked temperature increase was recorded by one of the two thermocouples in the left lower hepatized lobe which showed a final rise of 1.62°C. above the rectal temperature. A second thermocouple in this lobe showed an increase of only 0.6°C. above the rectal, similar to the change observed in the thermocouple in the opposite lobe.

In this experiment a careful histological examination of the lungs was made particularly with an eye to finding evidence of circulatory obstruction. Such evidence was indeed found. The microscopic examination was made independently by one of us (W. E.) who was not informed of the other experimental findings.

Result of Microscopic Examination of Lungs in Experiment D 48.—(Figs. 4 and 5.) The lungs were distended with Zenker's fluid and immersed in it. Sections were taken from all lobes and imbedded in paraffin. These were cut to a thickness of 5 to 6 micra and stained with eosin-methylene blue, iron-hematoxylin-eosin and Gram's stain. The findings are given below:

Right upper lobe: The section appears normal.

Right ventral lobe: The section appears normal.

Right lower lobe: One section appears normal. A second, however, at its center shows certain pathological changes. The bronchioles and alveoli are uniformly filled with an exudate consisting of alveolar epithelial cells and polymorphonuclear leucocytes. The epithelial cells are often filled with vacuoles and with Gram-negative bacilli, often also with polymorphonuclear leucocytes and erythrocytes. There is everywhere a proliferation of young epithelial cells from the alveolar walls and polymorphonuclear leucocytes are to be seen within the walls of the bronchioles and bronchi. The walls of the blood vessels are edematous in places. In the smaller vessels the adventitia frequently shows a proliferation of round cells. The capillaries are dilated with blood and the lymph vessels of the pleura are dilated with lymph. The exudate diminishes toward the peripheral portions of the section where the alveoli appear normal.

Left upper lobe: In the center of the section there is an area similar to the one just described, but smaller.

Left lower lobe: In three sections one sees the alveoli and bronchioles uniformly filled with exudate. There are no air-containing ones to be seen. The exudate has the same composition as described in the right lower lobe but is apparently greater in quantity. In some places where the exudate is plentiful there are comparatively few old, mostly phagocytosed erythrocytes. Here the capillaries are compressed and contain no red blood corpuscles (Fig. 5). Where there is less exudate, well preserved erythrocytes and some fibrin are often to be seen lying in the alveolar spaces. Here the capillaries are dilated with blood. The walls of the large vessels are edematous and infiltrated with many erythrocytes. The epithe-

lial lining of the bronchioli is mostly desquamated, that of the larger bronchi is well preserved. Many of the bronchial walls exhibit hemorrhages. The lymph vessels are dilated, containing erythrocytes, polymorphonuclear leucocytes and fibrin.

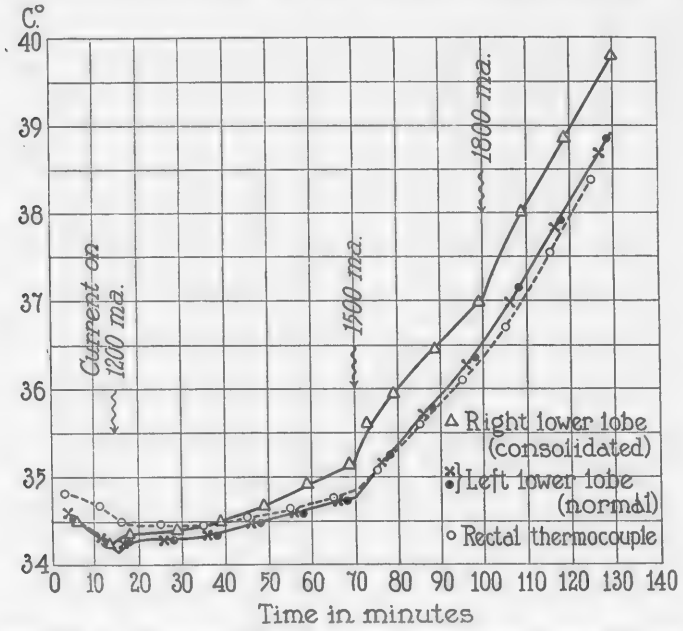
The significant finding is the compressed empty capillaries. Apparently this is due to the pressure of the exudate and depends for its occurrence upon the amount of exudate. This ischemic condition was found only in certain parts of the left lower lobe. One of the two thermocouples in this lobe showed a rise of temperature greater than that occurring in any of the other lobes. The second thermocouple, however, recorded an increase in temperature equal to that in the right lower lobe. The experimental findings are then quite consistent with the changes observed by microscopic study.

In Experiment D 49 the dog received 1 cc. per kilo of an actively growing culture of *B. friedlænderi*. Observations were made the following day. The dorsal half of the right lower lobe was consolidated. There was no local heating recorded by either of the two thermocouples imbedded in the pathological lobe. The final temperatures recorded in the consolidated portion of the right lower lobe were 37.75°C. and 37.87°C. after 42 minutes of diathermy at 1200 milliamperes, as compared with a temperature of 37.97°C. in the relatively normal left lower lobe. The rectal temperature recorded simultaneously by a mercury thermometer was 37.68°C. In this animal the microscopic picture of the right lower lobe (Fig. 6) showed the alveoli to be very irregularly filled with exudate. Air-containing alveoli were seen lying between those showing the inflammatory reaction. The capillaries, far from being compressed and empty, were dilated with blood. A similar dilatation of vessels, but no exudate, was found in the left lower lobe. It seems not improbable that the lack of local heating was associated with the absence of vascular obliteration.

A number of experiments was made, using cultures of Pneumococcus Type I in place of *B. friedlænderi* for intrabronchial insufflation. It was hoped that the fibrin reaction, usually stimulated by Type I pneumococcus, would cause a greater interference with the pulmonary circulation and therefore permit of more definite local heating. This was not the case. The response of dogs to intrabronchial insufflations of Type I pneumococci is an extremely variable one. They will often

tolerate large amounts of actively growing mouse-virulent cultures when injected by this route. We have selected for publication one experiment (D 54) in which a pneumonic consolidation was produced involving the caudal lobe and three-quarters of the right lower lobe, where a thermocouple had been placed.

The cut section of the right lower lobe was solid and apparently non-air-containing. Histological examination of the right lower lobe showed the alveoli and bron-



TEXT-FIG. 3. Experiment D 54. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

chioles mostly filled with exudate. In some places air-containing alveoli occurred amongst the others. The exudate consisted chiefly of polymorphonuclear leucocytes mixed with varying numbers of vacuolated alveolar epithelial cells often containing erythrocytes and degenerated cocci. Most of the capillaries were dilated with blood. Red cells were seen in the alveolar exudate and in some places hemorrhages were present in the walls of the bigger vessels. No evidence of capillary fibrin thrombi was to be found. The left lung was apparently quite normal though sections from the lower lobe showed that the vessels were dilated

with blood. Difference in temperature between the pathological and normal lobes was slight, but became more apparent on increasing the milliamperage to 1800 (Text-fig. 3). The final temperature in the consolidated lobe was 1°C. above the rectal temperature while the normal lung was 0.15°C. above the rectal temperature. The slight degree of elevation of temperature in the involved lobe produced by the current of 1200 milliamperes may be explained on the basis of no clear evidence of a grossly impaired circulation, since neither capillary thrombi were present nor sufficient exudate to compress the capillaries. For the higher current strengths it is probable that the existing circulatory disorder was sufficient to permit of a moderate degree of local heating.

The existence of obstruction to the circulation was further indicated by injection preparations made with the technique described by Gross (6). Satisfactory preparations could not be obtained at each attempt. The final picture depended upon the conditions prevailing during injection. The presence of puncture holes caused by the thermocouple needles made it impossible to use the lungs, in most of these experiments, for purposes of injection.

We present in Fig. 3 the photograph of a cleared dog's lung injected immediately after death with a barium-gelatin mixture. 2 days before, the animal (D 35) had been injected intrabronchially with 25 cc. of a broth culture of *B. friedlænderi*. It was killed by injecting 20 cc. of a saturated solution of $MgSO_4$ intravenously. The area of involvement described in the autopsy notes corresponds exactly to that portion of the lung which has injected poorly. We publish this photograph as further evidence for the impairment of the circulation in the pneumonic lung.

DISCUSSION.

We have deliberately chosen for publication only those experiments in which the proper combination of circumstances made it possible to demonstrate local heating of the lung by diathermy. These circumstances consist first of the development of a pulmonary lesion sufficient to cast a shadow when photographed by x-ray, and secondly of the accurate locating of thermocouples in both consolidated and normal lung tissue. When these conditions have been fulfilled we have been able to demonstrate local heating in three of four animals inoculated intrabronchially with cultures of *B. friedlænderi* and in one animal inoculated in a similar fashion with a culture of Pneumococcus

Type I. The variation of heat production in these selected experiments can be accounted for by the positions of the thermocouples and by the degree of impairment of the pulmonary circulation. The pathological process in these dogs' lungs presents three stages: (1) a stage of active congestion (Fig. 6) in which relatively little exudate is to be seen and the alveolar capillaries are dilated with blood; (2) a transitional stage (Fig. 4) in which the amount of exudate is increased and the erythrocytes lying in the capillaries have lost their regular shapes and usual staining properties, and finally (3) an ischemic stage (Fig. 5) in which all the alveoli are uniformly filled with exudate and the capillaries are compressed and free from erythrocytes. This final bloodless stage in which the local circulation is definitely impaired is the one in which we believe a certain degree of local heating with diathermy may occur.

We have purposely drawn no inferences as to heat production during diathermy in the lung of a patient suffering from pneumonia.

SUMMARY AND CONCLUSIONS.

1. An experimental pneumonia with more or less lobar distribution has been produced in dogs by the method of intrabronchial insufflation of *B. friedlænderi*, Type B, and Pneumococcus, Type I.

2. Such dogs as showed evidences of a pulmonary lesion when photographed by x-ray were selected for lung temperature measurements.

3. Measurements of lung temperature were made by means of thermocouples before and during diathermy.

4. The thermocouples which recorded the temperature in the consolidated lobes showed in most instances a more rapid rate of heating during diathermy than those in the normal lobes. The final increase in temperature in the pathological lobes over the normal lobes amounted to slightly more than 1°C.

5. When local heating occurred during diathermy it was of the order of magnitude found in a lung in which the branch of the pulmonary artery supplying it had been clamped.

6. Histological examination of the lungs showed the pathological reaction to consist of intraalveolar exudate composed of polymorphonuclear leucocytes and desquamated alveolar epithelium. In some

sections the exudate was sufficient to cause compression and emptying of the alveolar capillaries.

7. The local heating, we believe, depends upon this ischemic state of the smaller vessels.

8. Further evidence for an imparied circulation in the pneumonic lung is furnished by injection preparations in which the uninjected area corresponded exactly to the gross pathological lesion.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. X-ray photograph of thorax of Dog D 36 taken 42 hours after intrabronchial insufflation with culture of *B. friedlænderi*. The picture shows a slight shadow at the base of the left lung.

FIG. 2. X-ray photograph of thorax of Dog D 38, taken 26 hours after intrabronchial insufflation with culture of *B. friedlænderi*. The picture shows a slight shadow at the base of the right lung.

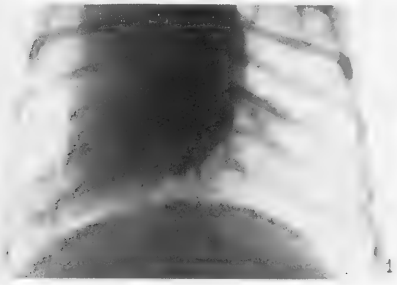
FIG. 3. Experiment D 35. Photograph of cleared barium-gelatin injection preparation of dog's lung in which consolidation had been produced by the intrabronchial insufflation of a culture of *B. friedlænderi*. The uninjected area corresponded to the part of the lung which showed gross pathological changes at autopsy.

PLATE 35.

FIG. 4. Experiment D 48. Left lower lobe. Magnification × 400. The photograph shows the transitional stage in which the erythrocytes in the capillaries have become irregular in shape and have lost their usual staining properties. Methylene blue and eosin.

FIG. 5. Experiment D 48. Left lower lobe. Magnification × 400. This represents the final ischemic stage. All the pulmonary alveoli are uniformly filled with exudate, the capillaries being compressed and bloodless. Methylene blue and eosin.

FIG. 6. Experiment D 49. Right lower lobe. Magnification × 400. The photograph represents the stage of active congestion in which the alveolar capillaries are dilated with blood. Methylene blue and eosin.



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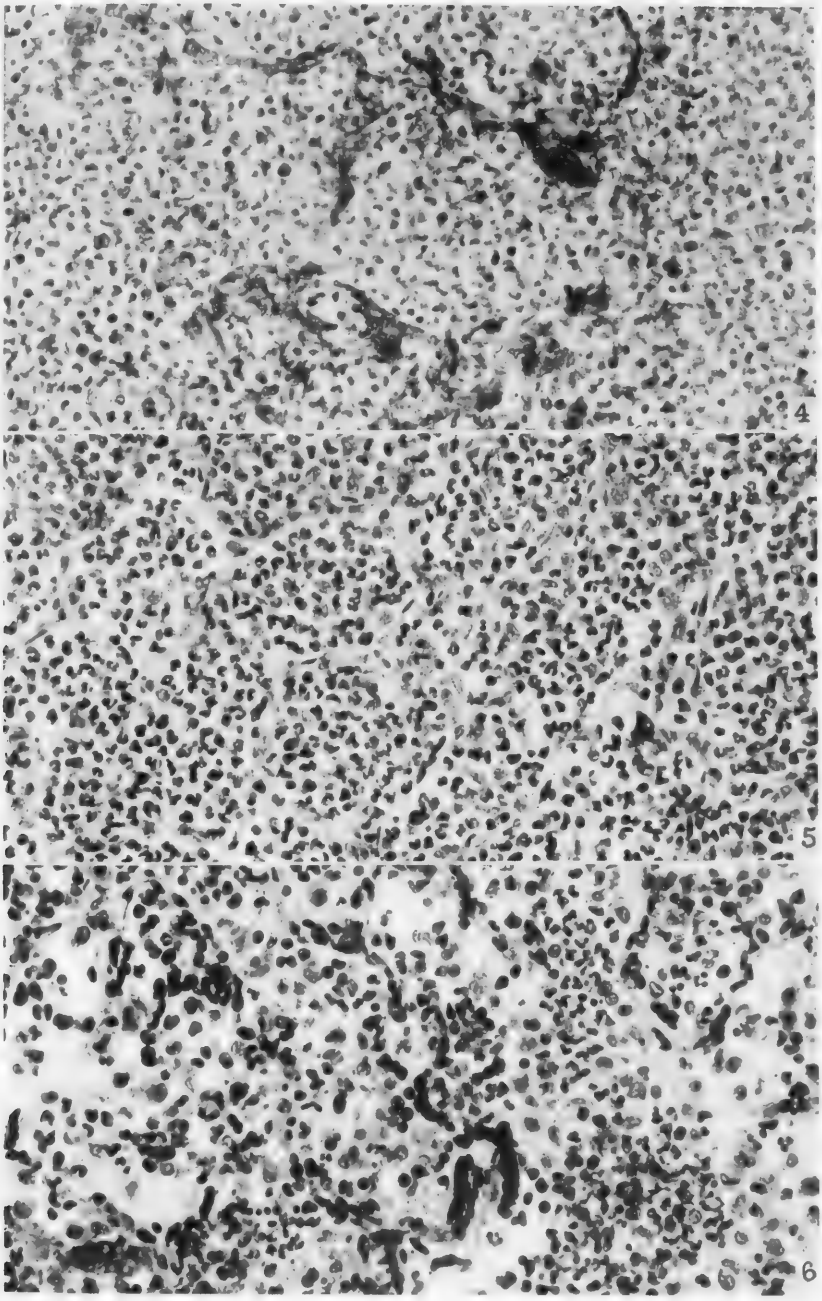

Photographed by Louis Schmidt.

(Christie, Ehrich, and Binger: Diathermy. V.)

ON INDIVIDUAL DIFFERENCES IN HUMAN BLOOD.*

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The existence of individual differences in human blood was shown long ago by means of normal isoagglutinins (1). Soon thereafter variations were found also in the blood of animals (goats) through the use of immune sera prepared by injecting goats with blood of different individuals of the same species (2). These observations were later extended to other animals.

Curiously enough the reactions with normal human isoagglutinins do not occur in a, so to speak, haphazard manner but they separate the human bloods into four sharply defined groups designated as O, A, B, and AB. (1, 3).** It seems superfluous to describe the well known properties of these four groups.

In contradistinction to the simple scheme encountered with human blood, are the findings of Todd and White (5) who studied the serum of cattle immunized against cattle plague with the blood of infected animals. Taking advantage of the isohemolytic properties of such sera they discovered a remarkable variety in cattle blood; and similar conditions have been observed in the blood of chickens (Landsteiner and Miller (6)).

Attempts have been made also to discover further differences in human blood in addition to the group distinction. Evidence along this line has been furnished by von Dungern and Hirschfeld (7) in experiments with absorbed normal animal sera. It is difficult however to apply this method to a systematic study and the work was not carried on further. Following the technique of von Dungern and Hirschfeld we found some differences (see also Landsteiner and

* See the preliminary reports in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 600, 941.

**In the present paper the nomenclature adopted by the American Association of Immunologists is used (4).

Witt (8)) aside from the conspicuous ones characterizing the groups, or those ascribable to the subdivision of group A, or due to the agglutinable factor found by Schiff (9) in blood cells of group O (*cf.* Witebsky and Okabe (10), Hirszfeld (11)). Our experiments were not regularly successful and frequently variations of smaller order could not be confirmed on repetition of the tests.

Another way of showing distinctions among individual bloods but also somewhat difficult of application, was found in the reactions of "cold agglutinins" of normal human sera (12–14).

The present paper concerns itself with a method of differentiating human bloods which yielded clear-cut and reliable results. It is based upon the use of immune agglutinins.

Using this technique Hooker and Anderson (15) found that immune sera produced in rabbits by injection of blood of group O still contained agglutinins for O blood after absorption with cells of any other group. The authors were inclined to explain this effect on the assumption of a property common to group O bloods.

EXPERIMENTAL.

Our first observations were made in experiments with stock anti-human blood immune sera from rabbits. Out of forty-one sera four were found that, after exhaustion with one sample of human blood, still contained agglutinins acting on a majority of bloods of all four groups while other bloods were not agglutinated. These tests showed the existence of an agglutinable property unrelated to the isoagglutinogens A and B, and differing from the latter in that there was not found a corresponding isoagglutinin in human serum. Naturally endeavors were made to produce immune sera endowed with the peculiar property described, by injecting rabbits with bloods possessing the new quality which may be designated as M. This was found to be rather difficult because only a few of the rabbits produce potent sera specific for M. However, on immunizing a sufficient number of animals, several such sera were obtained.

Some of the immune sera exhibited a different effect. When they were absorbed with blood of the type M+, the supernatant fluid reacted intensely and selectively on certain blood specimens, thus revealing a second agglutinable property (N).

The production of antibodies for N by injecting positively reacting bloods succeeded easily and some such sera were found among our supply of anti-human blood immune sera.

As was to be expected, anti-N agglutinins were not found in normal human sera. Also in normal animal sera we have not yet detected agglutinins for M or N.

An immune serum for a third agglutinable factor P was prepared by injecting blood (of colored individuals) selectively acted upon by absorbed normal rabbit and beef serum (according to the method of von Dungern and Hirschfeld).

In order to prepare specifically reacting agglutinin solutions, the inactivated immune sera in a dilution of 1:15 to 1:30 were treated with half the volume of packed, washed blood cells lacking the respective agglutinogens. A second treatment with the same or a smaller quantity of blood was required ordinarily to remove completely the agglutinins acting on human blood in general. The mixtures were allowed to stand for 1 hour at room temperature and were centrifuged. The fluids for N were prepared at first in this manner; subsequently, as will be explained below, the mixture of blood and serum was kept for ½ to 1 hour at 37–40°C.

The details of the procedure have to be determined in preliminary experiments and the absorbing blood must be selected with regard to the properties of the serum, *e.g.*, the presence of group agglutinins. Before setting up the main experiments the fluids were controlled by testing them with known bloods.

The tests were made by adding to 3 or more drops of the agglutinating fluids 1 drop of 2.5 per cent suspension of washed blood. The readings were made after the tests had stood for 2 hours at room temperature, or 1 hour at 37°C., if the fluids had been prepared at this temperature. The strength of the reaction is indicated as follows: F. tr. = faint trace; tr. = trace; ±, +, +±, etc. + signifies clumps visible without magnification or with a hand lens (magnification 6×) or clumps of medium size seen in the microscopic field (magnification 100×); ++ signifies large clumps seen with the naked eye and +++ complete agglutination.

For the production of immune sera freshly drawn and citrated blood (mostly of group O), after washing, was injected into rabbits at weekly intervals. The first injection of 3 cc. was given intravenously; the following injections of 4 cc. each, intraperitoneally. The sera were tested by absorption 6 days after the third and each subsequent injection. The animals were bled (mostly after four or five injections) the day following the tests when the sera had a sufficient content of the desired antibodies, i.e., when they gave powerful specific reactions after absorption. For the preparation of anti-M immune sera it seems preferable to inject bloods of the M+ N− type.

TABLE I.

The immune sera diluted 1:20 were absorbed with suitable blood lacking the particular agglutinogen, as described. The N agglutinins were prepared by absorption at room temperature (see page 769). Readings were made after 2 hours at room temperature.

Blood No.	815	816	817	819	821	822	823	824	825	826	828	829	830	831	832	833	834	835	836	837	838	839	840	841
Group	O	O	O	B	O	O	B	O	B	O	O	B	O	A	O	A	O	O	O	O	A	O	A	O
Immune serum 1; agglutinins for M	++±	+±	++±	++±	++±	0	++±	0	+++	++±	+++	0	0	+++	++±	+++	++±	+++	+++	+++	+++	++±	++±	0
Immune serum 18; agglutinins for N	0	tr.	0	+±	++	++±	0	+++	++±	±	++	++±	++±	+	+±	0	++	0	+±	++	0	++	+±	++±
Immune serum 5; agglutinins for P	+±	++	++	++±	+±	+±	±	+	++	+	+	+	0	++	+±	±	0	+	+	±	+	+	0	0

It was of considerable importance to have at one's disposal a number of individuals whose blood could be examined repeatedly. The work was facilitated also by keeping particular specimens in a mixture recommended by Rous and Turner (5 volumes of 5.4 per cent glucose solution, and 2 volumes of a 3.8 per cent sodium citrate solution, for 3 volumes of blood) (16). In this solution the erythrocytes were still agglutinable when the blood was kept sterile in the refrigerator for several weeks.

TABLE II.

Frequency of M in the Four Blood Groups.

Group		O		A		B		AB		Total Number	
Reactions for M		+	−	+	−	+	−	+	−	+	−
Men	Number	299	64	285	48	114	21	34	15	732	148
	Percentage	82.4	17.6	85.6	14.4	84.4	15.6	69.4	30.6	83.2	16.8
Women	Number	115	40	80	17	32	10	13	3	240	70
	Percentage	74.2	25.8	82.5	17.5	76.2	23.8	81.2	18.8	77.4	22.6
Total Number		414	104	365	65	146	31	47	18	972	218
Percentage		79.9	20.1	84.9	15.1	82.5	17.5	72.3	27.7	81.7	18.3

TABLE III.

Frequency of N in the Four Blood Groups. Absorptions and Tests at 37°C.

Group	O		A		B		AB		Total No.	
Reactions for N	+	−	+	−	+	−	+	−	+	−
Total No.	162	48	143	46	28	10	6	3	339	107
Percentage									76.0	24.0

A representative experiment on twenty-four blood samples taken at random with exhausted immune sera containing agglutinins for M, N, and P, respectively, is given in Table I.

The frequency (in white individuals) of the positive and negative reactions for the property M and their occurrence among the blood groups are presented in Table II. The distinction between positive and negative bloods for M was regularly sharp when the exhaustion was made with suitably selected bloods.

It is seen from the table that positive reactions are much more frequent than negative ones. The percentage figures for the four groups do not deviate greatly from the total average except for group AB, but here the number of individuals examined is too low to warrant any conclusion. A similar remark may apply to the figures for the sexes.

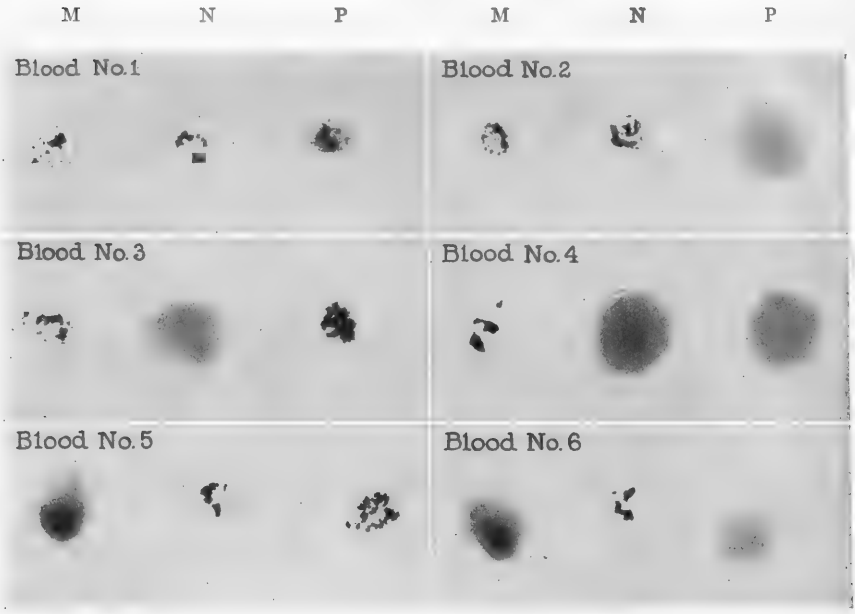

FIG. 1. Six selected bloods of group O tested with agglutinins for M, N, and P; natural size.

The distribution of the property N among the four blood groups is summarized in Table III.

For P our present results do not permit of a similar statistical survey but in general they indicate that there is no characteristic group distribution.

From the presence or absence of three agglutinogens, M, N, or P, there would follow eight possible combinations. Of these six actually

have been found in groups O and A. In groups B and AB some of the rarer types have not yet been found, most likely because of the comparatively smaller number of specimens completely examined.

The six combinations observed are illustrated in the photographs (Figs. 1 and 2). The tests reproduced were made with blood from

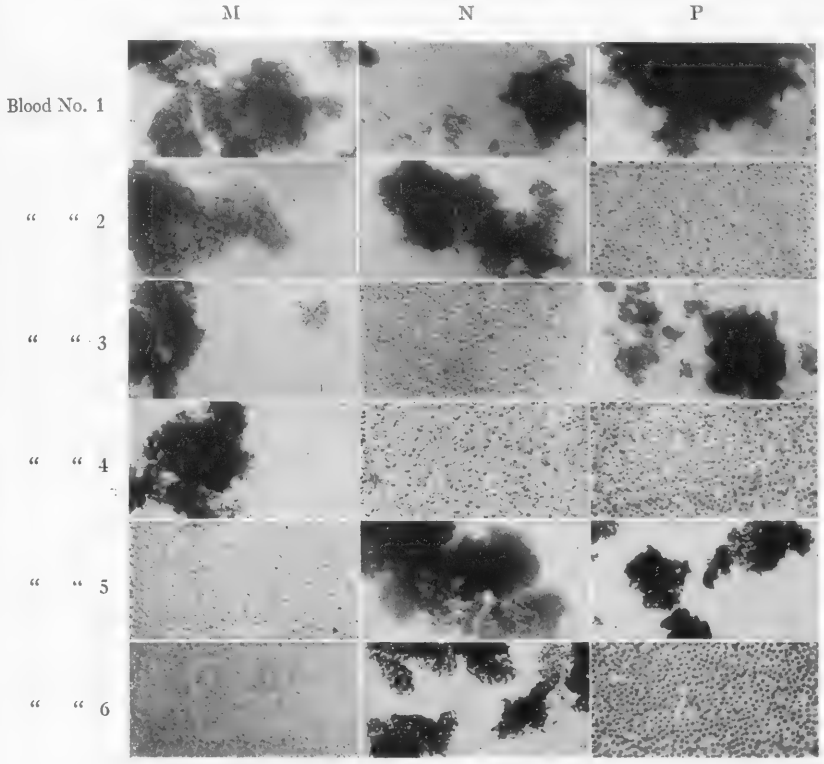

FIG. 2. The same tests as shown in Fig. 1, magnified 1:250.

six selected individuals all belonging to group O. The absorptions and tests for N were carried out at room temperature.

The two combinations not observed are those in which both properties M and N are absent. From the incidence of M− and N− bloods

TABLE IV.

Several immune sera absorbed with one M— blood (549). Sera 8, 21, and 22 diluted 1:15: Sera 1 and 20 diluted 1:20.

Agglutinins for M: serum No.	Group O								Group A									Group B		Group AB	
	201	205	235	244	252	262	266	279	199	220	242	500	549	566	568	570	591	592	23	560	272	522
1	+++	0	±	f. tr.	++±	+++	0	++	0	0	0	+++	0	0	++±	++	++±	++±	+	0	0	+++
8	++±	0	0	0	++±	++±	0	+±	0	0	0	++±	0	0	++±	++	+++	+±	+	0	0	++±
20	+++	0	0	0	+++	+++	0	++±	0	0	0	+++	0	0	+++	++±	++±	++±	+±	0	0	+++
21	+++	0	±	f. tr.	++±	+++	0	++±	tr.	0	f. tr.	+++	0	f. tr.	+++	++±	++±	++±	+	f. tr.	0	++±
22	++±	0	tr.	f. tr.	++±	++±	0	++	0	0	f. tr.	+++	0	0	+++	++	++±	+±	+	f. tr.	0	+++

TABLE V.

One Anti-M Serum Absorbed with Various M— Bloods.

Agglutinins for M prepared from serum 8 by absorption with various bloods			Group O						Group A								Group B				Group AB			
									A^2	A^1	A^2	A^2	A^1	A^1	A^1	A^2					A^1	A^1	A^2	A^2
Blood No.	Group	Subgroup	214	218	235	262	301	306	220	359	369	382	506	512	566	575	198	236	291	292	531	532	535	557
306	O		+++	+++	0	+++	0	0	f. tr.	+	±	++±	+++	+++	+	+++	0	0	0	++±	+	++±	+++	+++
359	A	A^1	+++	+++	0	++±	0	0	0	0	0	++	+++	+++	0	++±	0	0	0	++±	0	++±	++±	++±
566	A	A^1	+++	+++	0	+++	0	0	0	0	0	++	+++	+++	0	+++	0	0	0	++±	0	++±	++±	++±
220	A	A^2	+++	+++	0	+++	0	0	0	±	0	++	+++	+++	±	+++	0	0	0	++±	tr.	+++	+±	++
369	A	A^2	++±	++±	0	++±	0	0	0	f. tr.	0	++	++±	++±	f. tr.	++±	0	0	0	++	0	++	+±	++

one would expect an incidence of about 4 per cent bloods lacking both properties. This discrepancy evidences a negative correlation between the two agglutinable properties M and N, a conclusion supported by the fact that invariably M− bloods reacted intensely with the N reagent.

To form a correct judgment of the significance of the phenomena it was of importance to examine in how far they are influenced by variations in the reagents. In the first place a comparison was made of several anti-M immune sera in order to establish whether we were dealing with a definite agglutinable property or whether the results change contingent upon the special serum employed. The following experiments in which five anti-M immune sera were absorbed with the same blood specimen furnish proof for the first alternative (Table IV).

It is evident from the table that the strongly positive reacting bloods are the same regardless of the serum employed. In no case is the reaction of a blood intensely positive with one anti-M serum and negative with another. Identical results have since been obtained with several additional immune sera. Whether there is any disproportion in the strength of the reactions when M+ bloods are tested with a number of immune sera we are unable to decide as yet. With the sera 8 and 20 the reactions are almost uniformly strong or entirely negative. The remaining sera showed slight or weak reactions with some bloods which, doubtless, according to the other tests, lack the property M. These will be discussed presently.

In a second series of tests (Table V) one of the sera, No. 8, was absorbed with five different bloods, one of group O and four of group A, two of which belonged to subgroup AA^1 and two to AA^2*.

This experiment agrees with the former, for, no matter which blood was used for the preparation of the fluid, the strong reactions occurred with the same blood specimens. Hence it follows that all these reactions involve one sort of agglutinogen and its corresponding agglutinin.

The fluid prepared by absorption with blood of group 0 gives weak or moderate agglutination effects with all the bloods of group A or AB, evidently due to the presence in the immune serum of an agglutinin for A. These reactions are removed by absorption with blood AA^1 while after treatment of the immune serum with blood AA^2 there is still some agglutination with bloods AA^1 (or AA^1B).

* For the nomenclature of the subgroups see (8, 14).

Similar interfering reactions were encountered not infrequently throughout these studies. In general they were brought about by the presence in the sera of normal or immune antibodies for the group factors A and B, of antibodies acting by preference on bloods of group 0 (Schiff (9)) and by the coexistence in the same immune serum of more than one of the new agglutinins; besides there may be other agglutinogens not yet analyzed. Suitable absorption experiments serve to eliminate such additional antibodies.

TABLE VI, *a*.

Serum 20 diluted 1:15 was absorbed once for 2 hours at room temperature with half its volume of blood lacking the factor M. Some of the fluid obtained was removed and the remainder was divided into two equal parts. One of these two fractions was further absorbed three times at room temperature with half its volume of the M− blood, and the other was simultaneously treated in the same manner but with washed sheep blood; after each absorption a small amount of fluid was withdrawn for the tests. The last absorption was made at 0°C. and the fluid was obtained by centrifuging in a jacket containing ice and water.

The fluids of the first, fourth, and fifth absorptions were titrated in progressively doubled dilutions, using 3 drops of the liquid and 1 drop of 2.5 per cent suspension of M+ and M− blood. Readings were made after the tests were kept 2 hours at room temperature.

Fluid after	Absorbed with blood	Test with blood	Fluid diluted 1:				
			15	30	60	120	240
1st absorption	Human M−	M+	+++	++	+	f. tr.	0
		M−	0	0			
4th "	Human M−	M+	+±	+	f. tr.	0	0
		M−	0	0			
	Sheep	M+	++±	++	+	0	0
		M−	0	0			
5th "	Human M−	M+	+	f. tr.	0	0	0
		M−	0	0			
	Sheep	M+	++±	++	+	tr.	0
		M−	0	0			

The question whether the properties M+ and M− are of a qualitative nature was approached by means of repeated absorptions of the immune sera with M− blood. In the experiment recorded in Table VI, *a*, and Table VI, *b*, it was found that the antibody for M could

be gradually absorbed from serum 20, the greatest effect being obtained at low temperature while in serum 72, even after several absorptions, there was only an indifferent diminution of the specific agglutinin, not more marked with human than with sheep blood which was used as a control. Consequently the property M is characterized as a particular agglutinogen according to serological terminology.

Some anti-M sera were found whose specificity could be recognized by different degrees of agglutination when the exhausted sera were tested with the two sorts

TABLE VI, *b*.

An experiment similar to the preceding was made with anti-M immune serum 72. This serum diluted 1:30 required two absorptions with human M− blood for removing the common agglutinins. After withdrawing some of the fluid it was divided in two portions and the experiment was continued as above with human blood and sheep blood. The last absorption was carried out at 0°C. The fluids of the second, fourth, and fifth absorptions were tested as before.

Fluid after	Absorbed with blood	Tested with blood	Fluid diluted 1:				
			30	60	120	240	480
2nd absorption	Human M−	M+	+++	+++	+	+	0
		M−	0	0			
4th "	Human M−	M+	+++	++	+	+	0
		M−	0	0			
	Sheep	M+	+++	++±	+±	+	0
		M−	0	0			
5th "	Human M−	M+	++	+±	±	f. tr.	0
		M−	0	0			
	Sheep	M+	++	+±	±	0	0
		M−	0				

of corpuscles; they were not good for further work because the species agglutinins could not be removed without a simultaneous loss of the specific action.

The action of several (six) anti-N immune sera on a series of bloods and the effect of the exhaustion at room temperature of two immune sera with various bloods were studied in an analogous manner as

described for the property M. Since the strong reactions occurred always with the same bloods the experiments warrant the assumption that here, too, a definite serological factor comes into play. This factor may be subject to some variation as will be discussed presently.

A difficulty was encountered, owing to the fact that on treating anti-N immune sera several times with N- bloods, there was a rather rapid diminution of the agglutinins for N. As a consequence it was not easy to estimate the adequate degree of absorption, although fluids of marked specificity could be prepared repeatedly (see Table I).

TABLE VII.

Each of four anti-N immune sera diluted 1:20 were absorbed three times with one-half volume of pooled blood of four individuals of group A lacking N. One set of the absorptions was performed at room temperature and the other at 37° (water bath). In the latter case the fluids were separated by centrifuging for about 1 minute at high speed in a jacket of warm water (about 50°): At the end of the centrifuging the temperature of the water was 37° or but little below. The fluids were tested with six selected bloods of group A, two of which reacted negatively, two moderately, and two intensely.

Absorptions and tests at room temperature

Anti-N immune serum	Fluid after	Blood No. 806	1010	546	931	851	953
18	1st absorption	tr.	±	++±	++±	+++	+++
	2nd "	0	0	±	tr.	++	++
	3rd "	0	0	0	0	±	±
22	1st "	0	0	++	++±	+++	+++
	2nd "	0	0	±	±	++±	++
	3rd "	0	0	f. tr.	0	+	±
26	1st "	f. tr.	f. tr.	++	++±	+++	+++
	2nd "	0	0	++	+±	++	+++
	3rd "	0	0	+±	+	++	++
61	1st "	tr.	0	++±	+++	+++	+++
	2nd "	0	0	±	+	++±	++±
	3rd "	0	0	tr.	f.tr.	+	+

TABLE VII—*Concluded.*

Absorptions and tests at 37°

Anti-N immune serum	Fluid after	Blood No.					
		806	1010	546	931	851	953
18	1st absorption	++	+±	++±	+++	++±	++±
	2nd "	f. tr.	0	++±	++±	+++	+++
	3rd "	0	0	++	+±	++±	++±
22	1st "	±	+	++	+++	+++	+++
	2nd "	0	0	++	++	+++	++±
	3rd "	0	0	+±	+	++±	++±
26	1st "	++	+±	++±	++±	+++	++±
	2nd "	+±	+	++±	+++	+++	+++
	3rd "	0	0	++±	++±	+++	++±
61	1st "	++	++	+++	+++	+++	+++
	2nd "	+	±	++±	++±	+++	+++
	3rd "	tr.	f. tr.	+±	++	+++	+++

The technic was improved by carrying out the absorptions and also the tests at 37° or 40° C.* Under these conditions the N antibodies are diminished by repeated absorption with N- bloods at a slow rate (Table VII) and the results are generally satisfactory. On random selection of anti-N sera and absorbing bloods, also weak or moderate reactions are apt to occur with bloods that react negatively when other immune sera or absorbing bloods are chosen. This may be due to quantitative or qualitative variations in the agglutinogen N aside from other reactions as discussed above for the property M (p. 766).

The agglutinable property designated as P has not been studied extensively. Doubtless the reactions as presented in Table I are different from those for M and N and are independent of the group agglutinogens A and B; furthermore we found a characteristic distribution of P in white and colored individuals.** But it has not been

* With this method, incidentally, a considerable proportion of stored antihuman blood immune sera were found to contain smaller or larger fractions of N agglutinins.

** See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941.

established that parallel results can be obtained with several immune sera which would define a single quality.

A serum of an individual in group B containing an abnormal isoagglutinin was recently described by Ottenberg and Johnson (17). Having had the opportunity to examine this serum, we found in conformity with the authors named, agglutination reactions (of moderate to weak intensity) with numerous bloods of groups O and B. By absorbing this serum with certain bloods A, it was possible to show that the abnormal isoagglutinin acted also on the majority of group A bloods.

These isoagglutination reactions do not coincide with the reactions for M, N, or P.

The observations described open the possibility of making an individual diagnosis of human blood for forensic purposes in cases in which this could not be done hitherto. In preliminary experiments the properties M and N could be demonstrated in blood kept in a dry state (on glass) for several weeks. The method consisted in absorbing specifically reacting fluids with stromata prepared from small amounts (50 mg.) of the dried blood.

Immunization Experiments.—To determine whether the formation of the antibodies described, depends solely on the individuality of the animal used or also upon the antigen injected, the following experiment was carried out: 12 rabbits were injected with blood of the type M+ N− and 6 with blood of the type M− N+, all belonging to group O. Out of the 12 animals after four injections of M+ N− blood there were 1 strong, 1 weak anti-M immune sera, and 5 of rather moderate strength. Two of the latter became strongly active after one or two additional injections. In this series there were 2 or 3 which had a weak action for N. (After a further injection 2 other animals receiving M+ N− blood developed antibodies for N, 1 of moderate and 1 of weak activity.)

Of the 6 animals receiving M− N+ blood all developed immune sera specific for N; 4 reacted strongly and 2 moderately; none of these contained antibodies for M.

The experiment shows that potent antibodies were obtained when the homologous agglutinogen was injected but that antibodies of weak activity were also produced by animals which had not received the corresponding antigen. These observations are not exceptional

and similar cases were reported, *e.g.*, by Weil and Felix (18) and Furth (19) with bacilli of the typhoid group (*cf.* Halber and Hirszfeld (20)).

Tests with Blood of Anthropoid Apes.—The presence in the blood of apes of agglutinogens indistinguishable from the human group fac-

TABLE VIII.

*Tests for M in Blood of Primates.**

Anti-M immune serum absorbed with human blood	Chimpanzees										Ourang		Gibbons					Man	
	1	2	3	4	5	6	7	8	9	10	1	2	1	2	3	4	5	M−	M+
M−	+±	++	++±	+±	+±	+±	+	++	++	++	+±	+	0	0	0	0	0	0	++±
M+	0	0	0	0	0	0	0	0	0	0	+±	+	0	0	0	0	0	0	0

* The blood of the first 6 chimpanzees, Ourang 1, and Gibbon 1 were examined at the same time.

TABLE IX.

Tests for N in Blood of Primates. Absorptions and Tests Were Made at 37°C. Serum 18 Was Tested after 2 Absorptions (18a) and 3 absorptions (18b).

Absorbed with blood	Immune anti-N serum No.	Chimpanzees				Ourang	Man	
		3	4	5	10		N+	N−
N−	12	tr.	tr.	tr.	tr.	+±	++±	0
	18a	++±	++	++±	++±	++	+++	f. tr.
	18b	+	+	+±	+±	+±	+++	0
	22	+	+	0	+	+	++±	0
N+	12	0	0	0	0	+±	0	0
	18a	0	±	±	0	++	tr.	0
	18b	0	f. tr.	tr.	0	+±	0	0
	22	0	f. tr.	0	0	+	0	0

tors A and B has been shown previously (Landsteiner and Miller (21), *cf.* von Dungern and Hirschfeld (7)). Some experiments were made to establish whether also the new agglutinable properties are to be found in the blood of anthropoids. To account for the existence of agglutinins in the blood of apes that cannot be removed by human

cells,* absorptions had to be made with bloods both lacking and possessing the factor in question; a positive reaction was indicated when agglutination took place in the first but not in the second instance.

The tests for P were negative in the blood of chimpanzees and 1 ourang. As to the quality M, it appeared to exist in the erythrocytes of each of 10 chimpanzees, but not in the blood of 5 gibbons (1 *Hylobates lar*, 3 *Hylobates leuciscus*, 1 *Symphalangus syndactylus*) and 2 ourangs (Table VIII). The reactions for N in the blood of chimpanzees were distinctly positive with one of the immune sera, but moderate or faint with two other sera tested (Table IX).

That the properties M and N in the blood of chimpanzees and man are related though not entirely identical is seen from the results with the various anti-N immune sera and from the observation that one anti-M serum very active for human blood acted on chimpanzee blood positively but markedly less so than the other sera.

Of the lower mammalians and birds there were examined for the property M: 2 macacus rhesus, 2 vervets, 1 baboon, 1 sapajou, 1 lemur; 1 horse, 4 cattle, 1 sheep, 2 pigs, 1 dog, 1 cat, 25 rabbits, 2 guinea pigs, 2 rats, 1 mouse; 1 duck, 1 chicken, and 1 pigeon. For N only rabbits, 23 in number, were examined (absorptions at room temperature). The tests gave negative results.

DISCUSSION.

In the present studies a method is described which led to the detection of well defined individual differences in human blood in addition to those characterizing the blood groups. On repeated examination of the same individuals the properties were constant. The reactions observed indicate the existence of distinct agglutinable properties. This is substantiated by the fact that for M there are no transitions between positive and negative reactions, since fluids with a titre of 1:64 for M+ blood did not react on M− blood; also blood negative for M has practically no affinity to the antibody of certain anti-M immune sera as shown by absorption experiments at room temperature.

With the two agglutinable properties N and P, an appreciable ab-

* See the tests with ourang blood.

sorption effect is brought about at room temperature also by blood negative in the agglutination test. The phenomenon can possibly be explained on the assumption that the antibodies for the particular agglutinogen and those for human blood in general are not entirely segregated but are partly in some sort of combination. This view is supported by the fact, already mentioned, that certain anti-M sera behave similarly while others stand repeated absorptions. There are other cases which may call for an analogous explanation. It has been shown, f. i., that from some normal human sera of group O, corpuscles A or B absorb not only the homologous but also a part of the heterologous isoagglutinins (8). Similarly agglutinin α^1 of human sera O and B can be removed by bloods of group A which lack A^1, particularly when the absorptions are made at low temperature.

The ultimate significance of the factors determined by serological reactions is still a matter for discussion and it is not at all certain whether to each factor there corresponds a special compound that might be isolated chemically (14). But there is evidence from a study of families that the agglutinable factors M and N are constitutional properties that are inherited as Mendelian characters.* As to their antigenic nature it is true that the immunization depends largely upon the individual response of the animal but, even so, the experiments indicate that the antibodies can be formed as a result of specific antigenic action.

The division of human blood into only four well defined blood groups was not in harmony with the manifold individual variations that become evident from the experiences on transplantation of normal tissues and tumors. Thus there was some reason to presume that the serological differences of cells and the transplantation specificity are phenomena of a different nature. This gap seems to be bridged by some previous findings (7, 14) and the present studies.

The six types aforementioned, if present, as is likely, in each of the four blood groups and in the subgroups of groups A and AB, differentiate 36 varieties of human blood. This number does not include the variations in the strength of the reactions which may also be determined constitutionally and it is improbable that we succeeded

* See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941, and unpublished results.

in detecting all differences which can be demonstrated by means of antibodies derived from rabbits. Possibly other animals when injected with human blood would furnish sera with new specific qualities. As stated already, with a reagent of a different sort, namely an abnormal isoagglutinin in a particular group B serum (Ottenberg and Johnson (17)), reactions were obtained which did not run parallel with those shown by the rabbit immune sera. To be sure this reagent is not available for general use, but still it further doubles the number of human blood varieties that can be differentiated.

Summing up all the known observations on the subject one is led to the opinion that almost every individual human blood may have its characteristic serological features (see Todd and White (5)) as already conjectured by von Dungern and Hirschfeld, although at present there is no actual method which would permit of an individual diagnosis of human blood. Conceivably this end could be achieved by the use of immune isolysins.

The results of studies concerning the heredity of the agglutinable properties and their distribution in populations of different racial composition are reserved for subsequent communications.

The findings dealt with have thus far no direct bearing on the selection of donors in transfusions because of the absence of corresponding agglutinins in normal human sera for the new agglutinogens.

SUMMARY.

A clear-cut differentiation of human blood, aside from the blood groups, could be made by means of special agglutinating immune sera. The observations point to the existence of several agglutinable factors for which no agglutinins are demonstrable in normal human sera. In view of the latter circumstance the results reported do not imply any change in the scheme of the four blood groups.

The body of serological evidence leads to the inference of a high degree of biochemical differentiation among individuals.

Again we are indebted for material used in this study to Dr. C. Floyd Haviland, Superintendent, Drs. I. J. Furman and John R. Knapp, First Assistant Physicians, and Miss Frances W. Witte, Superintendent of Nurses, of the Manhattan State Hospital, New York City.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

X. Litmus Constituents as Vital Stains: Their Preparation and Relative Usefulness.

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The recent use of litmus as a vital stain for mammals (1–3) has emphasized the need for a more precise knowledge of the nature of this dye. We have therefore undertaken the separation and investigation of the coloring matters comprised in commercial litmus with special reference to their use as vital stains.

Rous (1–3) has described in detail the character of the staining obtained in rats and mice after intraperitoneal injections of litmus solutions. The animals tolerate the dye, which colors them markedly, and they may remain stained for many weeks. Some of the tissues are colored diffusely, while the hue of others is traceable to an intracellular segregation of pigment. The observed differences in hue at different situations has great interest since they may be taken to indicate local differences in reaction.

The material used by Rous was prepared from Kahlbaum's cube litmus by extraction with hot water, treatment with acetic acid, and precipitation with alcohol, according to a method given by Sutton (4), similar to one originally described by Mohr (4–6). It contained therefore, according to the statements in the literature, at least three important dyes: erythrolitmin, azolitmin, and erythrolein.

As early as 1840, Kane (7–10) isolated from commercial litmus four constituents: azolitmin, erythrolitmin, erythrolein, and spaniolitmin. He considered the two first mentioned to be the essential colorimg matters of the indicator. From time to time other authors have reported the isolation of slightly different pigments, Wartha (11–13) finding what he termed indigotin, and Scheitz (12, 13) a blue coloring matter distinct from azolitmin. On the whole, however,

workers agree that azolitmin and erythrolitmin are the chief sensitive indicator substances present in commercial "litmus," that erythrolein, a relatively insensitive indicator pigment, is always present, while very rarely spaniolitmin is to be found as well, which last can be distinguished from azolitmin only with great difficulty.

Preparation of "Whole Litmus" from Crude Litmus Cubes.

Nearly all of the methods employed for the purification of litmus eliminate from the crude material merely its grosser impurities, notably gypsum. They yield a substance applicable to chemical needs but too complex for physiological experiments. The final product of the method of Mohr (5, 6), or of the procedures given in standard texts on analytical chemistry (4, 14), which we shall term "whole litmus," has been repeatedly analyzed by us and found to contain, as coloring matter, over 90 per cent of erythrolitmin, a little azolitmin, and some erythrolein. There is present in addition a very small amount of gypsum. It was "whole litmus" that Rous used to advantage as a vital indicator.

The "whole litmus" just referred to, while non-toxic when derived from some commercial samples, is highly toxic in other instances. In order to avoid the complication thus introduced, as well as to obtain more precise limiting conditions, we have deemed it best before passing to physiological studies, to isolate and test on animals the individual indicator substances. After trying several methods we have found it best to extract the pigments in the acid state, as follows.

Isolation of the Constituents of "Whole Litmus."

Kahlbaum's litmus cubes were ground to a fine powder, well extracted with hot water, and the insoluble matter removed by centrifugation. The extract was reduced to a convenient bulk by evaporation, and concentrated HCl was added drop by drop to decompose carbonates. When the solution had just turned red, and effervescence had ceased, the volume was measured and 4 cc. of concentrated HCl was added for each 100 cc. of extract. This was then placed on a water bath for 2 or 3 hours, that is to say until no further precipitation occurred. The whole was poured onto a filter paper and the precipitate washed free from acid with hot water and allowed to dry. The dried precipitate was transferred to an extraction thimble of a Soxhlet apparatus and extracted with ether until all soluble material had been removed. The ether was withdrawn

from the Soxhlet, and extraction continued with 95 per cent alcohol until the alcohol came away colorless.

In this way three pigments were isolated. The etherial solution, when evaporated, yielded an oily red liquid, erythrolein. The alcoholic solution deposited, on evaporation, a bright red powder, erythrolitmin. The brownish red, insoluble residue which remained in the extraction thimble was azolitmin.

The erythrolein needed no further treatment. The filtered alcoholic solution of erythrolitmin was removed to an evaporating dish and carefully dried, washed with ether to remove any remaining erythrolein, and again dried. The azolitmin residue was purified by washing, first with ether, then with alcohol, and dried. Since the pigments in acid state are insoluble in water, and moreover, when powdered and dried do not keep as well as in the alkaline form, which is water soluble, they were converted to the latter. For this purpose thick suspensions were made in water of the dried, powdered pigments in the acid state. Sodium hydroxide in normal solution was added until the indicator became bluish. The material was then evaporated to dryness, and the pigment powdered and kept sealed. For the purposes of vital staining 2 to 5 per cent solutions were made up fresh when desired in 0.9 per cent sodium chloride solution or in water.

"Whole litmus" was prepared, according to the method of Mohr (6, 14), and the product, dissolved in 0.9 per cent sodium chloride solution, was also employed as a vital stain in experiments to be described below.

Characteristics of the Isolated Pigments.

General Characters.—The physical characteristics, solubilities, and colors of the pigment substances generally found in litmus, have been described in detail by previous authors (7, 12, 15). Erythrolitmin and azolitmin are of especial interest to the physiologist for the range of color change of these, which is from red to blue, lies within the presumptive pH range of many living cells. Erythrolein changes hue much less markedly, merely from red, on the alkaline side, to red-orange on the acid, at about pH 6.0 to 5.9. It is therefore only

of interest in that it complicates the use of the whole dye, litmus. In acid solution both erythrolitmin and azolitmin are a clear crimson-red, in alkaline solution blue. In the latter both are dichromatic, being a dark red-violet in very concentrated solutions.

Color Intensity.—The color intensities of erythrolitmin and azolitmin as prepared above are different. A given amount of erythrolitmin will color a solution more deeply than will an equal amount of azolitmin. This has been apparent from comparisons, in a Duboscq colorimeter, of equivalent concentrations of the two dyes, in the alkaline and acid forms, respectively. Both dyes had more than three times the color intensity of "whole litmus."

Colorimeter Readings. Acid Form.—Since the dyes are insoluble in water in the acid form, 0.025 per cent solutions in 10 per cent acid alcohol were compared. All the solutions were reddish orange, of so similar a hue as to afford a good color match.

Five 0.025 per cent solutions of azolitmin were made from specimens of the dye, each prepared from a different batch of litmus cubes. Read in the colorimeter against one another a noticeable variation in color intensity was found, the strongest solution possessing 1.5 times the intensity of the weakest. Similar solutions of erythrolitmin from five specimens of the dye, each from a different source, when compared with one another showed as considerable variations in color strength. A mixture of equal parts of all the azolitmin solutions, compared with a similar mixture of all the erythrolitmin solutions, showed the color strength of the latter to be 1.39 times that of the former.

Alkaline Form.—To study the color intensity of the two dyes in the blue state, comparisons of 0.025 per cent solutions in N/1 NaOH were made. The matching of color intensity was easy, despite the fact that the azolitmin was a slaty blue, and the erythrolitmin more azure.

Five alkaline 0.025 per cent solutions of the five specimens of azolitmin were compared in the colorimeter and so too were similar solutions of the erythrolitmin specimens. Of the alkaline azolitmin solutions the strongest possessed 1.6 times the color intensity of the weakest. Among the erythrolitmin solutions a slightly more uni-

form color intensity was found, the strongest being 1.4 times as intense as the weakest. Repeated comparisons of a mixture of equal parts of all the alkaline azolitmin solutions with a similar mixture of all the alkaline erythrolitmin solutions showed the erythrolitmin to be 1.45 times as strong as the azolitmin in color.

The Effect of Differing Concentrations of the Dye upon the Color in Vitro.

Owing to their dichromatism the dyes, erythrolitmin, azolitmin, and "whole litmus," may show colors varying with the concentration, a potential cause of error when they are used as indicators. The magnitude possible to such an error was investigated by comparing the colors of solutions of each of these indicators at differing concentrations. Solutions, varying in strength from 0.005 per cent to 0.05 per cent, were made up in Sörensen's phosphate buffer solutions (16) (M/15 primary potassium phosphate KH_2PO_4 and M/15 secondary sodium phosphate Na_2HPO_4, $2H_2O$), having a pH range from 5.5 to 8.04. In the case of all these indicators the diluted solutions were pure blue at pH 8.0, but with a tenfold increase in concentration appeared red-violet. A shift of the red, toward the alkaline side, had occurred corresponding to a change of 0.2 to 0.3 pH. On the acid side of the range, however, there was no observed difference in the color of the solution with differing concentration.

The degree of color change with alterations in the pH could not be so readily determined in extremely concentrated solutions of the dye as in dilute solutions, but it may, of course, be greater. In the employment of the indicators for tissue study, when they are often found concentrated in intracellular granules, one must take into account possible errors referable to differences in concentration of the dye. For this reason one cannot expect to obtain precise knowledge concerning the pH by the use of the dye. But in connection with this point,—and to anticipate slightly results to be described later,—it may be stated that after injection of the litmus indicators into the mammalian body the differences in color of the intracellular granules are so great that differences in concentration can be but a minor factor in determining them.

Salt and Protein Errors.

The presence of salt or protein in a solution may so affect the color range of indicators that they become of little value for determining its hydrogen ion concentration. The salt and protein errors of litmus and azolitmin have been studied in the past and found to be great (17); but the possibility presents itself that these errors may not be shared by erythrolitmin, the constituent of litmus employed for purposes of vital staining in the studies to be reported in a companion paper. We therefore determined the magnitude of the errors of this dye. These errors were found to be large. A specimen protocol follows which illustrates the magnitude of the salt error in solutions containing differing concentrations of sodium chloride.

TABLE I.

Concentration of NaCl	Apparent pH color	Actual pH (electrometric)	Difference (salt error)
per cent			
.2	7.21	6.99	.22
.4	7.21	6.93	.28
.8	7.21	6.85	.36
1.6	7.16	6.70	.46
3.2	7.12	6.58	.59
4.8	7.08	6.41	.65

Salt Error of Erythrolitmin.—Sörensen's M/100 phosphate buffer solutions were prepared, having a pH range from 5.5 to 8.34. To 10 cc. of each in a series of tubes of equal bore 0.3 cc. of a 0.1 per cent solution of erythrolitmin was added. In a companion series of tubes 5 cc. of M/50 phosphate buffer of pH 7.0 and 0.3 cc. of 0.1 per cent erythrolitmin solution were placed. To each of this latter series of tubes sufficient quantities of sodium chloride in 5 cc. of water were added to yield 0.2 per cent, 0.4 per cent, 0.8 per cent, 1.6 per cent, 3.2 per cent, and 4.8 per cent sodium chloride solutions in the total volume of 10.3 cc. The concentration of buffer in these tubes became therefore like that of the control series, M/100, and the amount of erythrolitmin present in all was similar. The addition of sodium chloride brought about a pronounced change of color, the solutions appearing more blue with each increase in the salt content, as compared with the controls. Electrometric determinations of the hydrogen ion concentration of all the solutions were then made. As the table given below shows, the solutions containing salt appeared bluer, that is to say, more alkaline than their true pH would warrant.

From this experiment it is obvious that the "salt error" of erythrolitmin is large. It is of importance to note that the error is toward the alkaline side, that is to say, in the opposite direction from the error of the phthalein indicators.

Protein Error of Erythrolitmin.—Erythrolitmin shares also the disadvantage of litmus and of azolitmin in that its protein error is large. This, like the salt error, causes the indicator to appear more blue in a solution containing protein than it should from the actual hydrogen ion concentration. For the experiments we employed blood plasma and egg albumin solutions. A specimen protocol will be detailed.

Sufficient M/15 phosphate buffer, pH 6.47, was added to clear rabbit plasma to give the mixture M/50 concentration of this buffer. Air was bubbled through for 30 minutes to remove CO_2 and then for another half hour a mixture of equal parts of air and hydrogen. The addition of a small amount of this plasma, 1 to 9 cc. of M/15 buffer solution, pH 6.9, containing erythrolitmin in the red-violet stage, caused a change in the color to a pure blue.

To determine the protein error of erythrolitmin it was deemed best to make up plasma-indicator mixtures in such a way that their final color would be neither blue nor red but violet, as is erythrolitmin alone in buffer solutions of hydrogen ion concentration between pH 6.9 and 7.4. Within this narrow range a change in color can be noted with each difference of 0.2 pH. Test solutions were therefore made up of a total bulk of 10.3 cc., in each of which was 0.3 cc. of 0.1 per cent erythrolitmin solution and varying amounts of M/100 phosphate buffer, pH 5.59, and of a mixture in equal parts of buffered plasma, M/50, and of distilled water free from CO_2 (see Table II). These mixtures contained, respectively, 10 per cent, 20 per cent, 40 per cent, 50 per cent, and 60 per cent of plasma and the concentration of buffer in all was the same, a shade less than M/100.

For color comparison a series of M/100 phosphate buffer solutions, having a pH range from 5.04 to 8.34, was used and to 10 cc. of each, 0.3 cc. of the 0.1 per cent erythrolitmin solution was added. Upon adding erythrolitmin to plasma one encounters in addition to the protein error, another error due to the salt present with the protein. To determine the share of this additional source of error, "control solutions" were made up, each containing in the same concentration of phosphate buffer the amount of salt presumably added to the "test solutions" with the plasma. This was done by employing Ringer's solution minus its sodium carbonate content, together with the necessary amount of M/15 phosphate buffer solution to bring the buffer concentration of the total mixtures to M/100. In this way "control solutions" were obtained which were nearly like the "test solutions" except in the respect that the latter contained protein. In order to find "control solutions" which matched the violet "test solutions," it was necessary to make up several series. For as the amount of plasma in the

TABLE II.

Erythrolitmin Color as Influenced by Blood Plasma Constituents.

1 "Test solution"	2 "Control solution" containing the plasma equivalent in salts	3 Color comparison with erythrolitmin in phosphate buffer	4 Error due to plasma (salt and protein error). pH in Column 3 minus pH in Column 1	5 Error due to the salt equivalent of the plasma. pH in Column 3 minus pH in Column 2	6 Error due to protein alone. pH in Column 4 minus pH in Column 5
1.0 cc. plasma, M/50 phosphate buffer content 1.0 cc. water 8.0 cc. M/100 phosphate buffer, pH 5.59 0.3 cc. 0.1 per cent erythrolitmin solution	1.0 cc. Ringer's solution 7.5 cc. water 1.5 cc. M/15 phosphate buffer, pH 6.9 0.3 cc. 0.1 per cent erythrolitmin solution	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.1	pH 0.76	pH 0.23	pH 0.53
10.3 cc. buffer concentration M/100 pH electrometrically 6.42	10.3 cc. buffer concentration M/100 pH electrometrically 6.95	pH electrometrically 7.18			
2.0 cc. plasma, M/50 phosphate buffer content 2.0 cc. water 6.0 cc. M/100 phosphate buffer, pH 5.59 0.3 cc. 0.1 per cent erythrolitmin solution	2.0 cc. Ringer's solution 6.5 cc. water 1.5 cc. M/15 phosphate buffer, pH 7.4 0.3 cc. 0.1 per cent erythrolitmin solution	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.7	pH 0.92	pH 0.23	pH 0.69
10.3 cc. buffer concentration M/100 pH electrometrically 6.67	10.3 cc. buffer concentration M/100 pH electrometrically 7.36	pH electrometrically 7.59			

3.0 cc. plasma, M/50 phosphate buffer content 3.0 cc. water 4.0 cc. M/100 phosphate buffer, pH 5.59 0.3 cc. 0.1 per cent erythrolitmin solution 10.3 cc. buffer concentration M/100 pH electrometrically 6.85	3.0 cc. Ringer's solution 5.5 cc. water 1.5 cc. M/15 phosphate buffer, pH 7.8 0.3 cc. 0.1 per cent erythrolitmin solution 10.3 cc. buffer concentration M/100 pH electrometrically 7.68	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 8.04 pH electrometrically 8.01	pH 1.16	pH 0.33	pH 0.83

"test solutions" was increased a greater and greater color shift toward the blue side occurred (see Table II).

The actual hydrogen ion concentration of all the solutions was determined electrometrically.

It was evident from such findings as are given in the table that the protein error of erythrolitmin is very large. When mixtures of the indicator are made with plasma or egg albumin, containing salt as well, the apparent error is still greater, for both salt and protein cause a change in the color of the indicator in the same direction, that is to say toward the alkaline side. As a result of this combined error a protein solution which is at about pH 7.0, that is to say, neutral, may appear frankly alkaline upon the addition of erythrolitmin, that is to say, blue.

When to 10 cc. of rabbit's plasma rendered CO_2 free and buffered as described above with sufficient M/15 phosphate buffer to give the mixture M/50 buffer concentration, 0.3 cc. of 0.1 per cent erythrolitmin solution was added, the color of the mixture matched that of erythrolitmin alone in phosphate buffer M/100 of pH 8.34. The actual pH of the protein mixture determined electrometrically was pH 7.20 and that of the buffer solution 8.19. The colorimetric reading was therefore in error by pH 1.0. In M/100 buffer solutions the color of erythrolitmin at pH 7.2 is red-violet, at 8.34 pure blue.

It has been noted elsewhere (1, 2, 3) that litmus segregated by living cells out of body fluids dyed blue with it is held in the red form. This is scarcely what one would expect were the intracellular reaction neutral or slightly alkaline for the influence both of protein and of salt would tend to render the litmus blue under such conditions. It is a matter of interest therefore to know the actual hydrogen ion concentration of protein mixtures rendered just sufficiently acid to bring the indicator to the red color it assumes in intracellular granules.

The protocol of one experiment on the point out of a number will be given.

To 3 cc. of buffered plasma, 1 cc. M/3 KH_2PO_4 solution (pH about 4.5) was added and 0.3 cc. of 0.1 per cent erythrolitmin solution. The resulting color was violet, not red. To the mixture phosphoric acid M/15 was added in just sufficient quantity (0.6 cc.) to bring about a brick red color like that of the erythrolitmin-stained granules frequently seen within tissue cells. The color of this

mixture matched that of erythrolitmin in M/15 phosphate buffer of pH 5.91. Electrometrically the pH of the plasma solution was found to be 4.81.

In connection with such findings it is to be recalled that the concentration error of erythrolitmin is in the opposite direction to that of the salt and protein errors, that is to say, the more concentrated the dye the more does it tend to appear red. It follows that an intracellular granule containing the dye in concentrated form need not have as great an acidity as that prevailing in the experiment above in order that it shall appear red.

From the foregoing results taken together it is obvious that in vital staining with erythrolitmin, one must content oneself ordinarily with the determination of relative differences in pH.

The Donnan Equilibrium Has No Effect upon the Distribution of the Litmus Derivatives.

A dye taken up by tissue cells becomes subject to the forces incident to the presence of protein substances. Our finding, that some changes in the color of the dyes result from changes in their concentration, made it important to study the influence of hydrogen ion concentration on the distribution of dye between fluid and protein, in mixtures of the two. A series of solutions were prepared containing powdered isoelectric gelatin and varying amounts of acid and alkali. "Whole litmus" or erythrolitmin was added to each. 20 hours later the fluid was separated by filtration and the amount of dye in it measured colorimetrically. All the solutions were found to contain the same proportions of the indicator. The conditions set up by the Donnan equilibrium did not change the relative distribution of the dye at the various hydrogen ion concentrations. From this finding it would seem that the probability of an error, caused by concentration changes due to the presence of protein, is slight.

A series of acid and alkaline mixtures were made up in 0.9 per cent NaCl solution, using N/1 HCl and N/1 NaOH in proper quantities to give N/5, N/20, N/40, N/80, N/160 to N/1280 acid in saline, and N/80, N/160, N/320, N/640, and N/1280 alkaline in saline. To 50 cc. of each of these 2 gm. of powdered isoelectric gelatin was added and 1 cc. of a 1 per cent solution of "whole litmus," just sufficiently alkaline to be in the blue state. The mixtures were each shaken 1 minute, packed in ice, and allowed to stand with the gelatin completely submerged in the fluid

for 20 hours. The litmus solution gave a red color to the gelatin and a blue to the supernatant liquid of the N/640 and N/1280 acid solutions immediately upon its addition to them; but after standing the color of the gelatin and supernatant liquid was uniform. The N/160 and N/320 NaOH solutions seemed to be close to the neutral point for the indicator, whereas the N/80 NaOH solution was alkaline to it, as shown by the distinctly blue color, and the N/640 NaOH solution acid, appearing red.

The supernatant liquids were separated from the gelatin by rapid suction filtration and the amount of dye present in each was estimated by adding to each 0.6 cc. of 40 per cent NaOH to convert the color to a strong blue to be read in a Duboscq colorimeter against a standard. For the latter purpose there was employed a solution containing 1 cc. of 1 per cent "whole litmus" to 50 cc. of saline with 0.6 cc. of 40 per cent NaOH. It will be seen that the amount of indicator equalled the total added to each of the gelatin mixtures.

The distribution of litmus was as follows:

Acid.

	N/80	N/160	N/320	N/640	N/1280
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
In supernatant liquid	54	53	52	47	49

Alkaline.

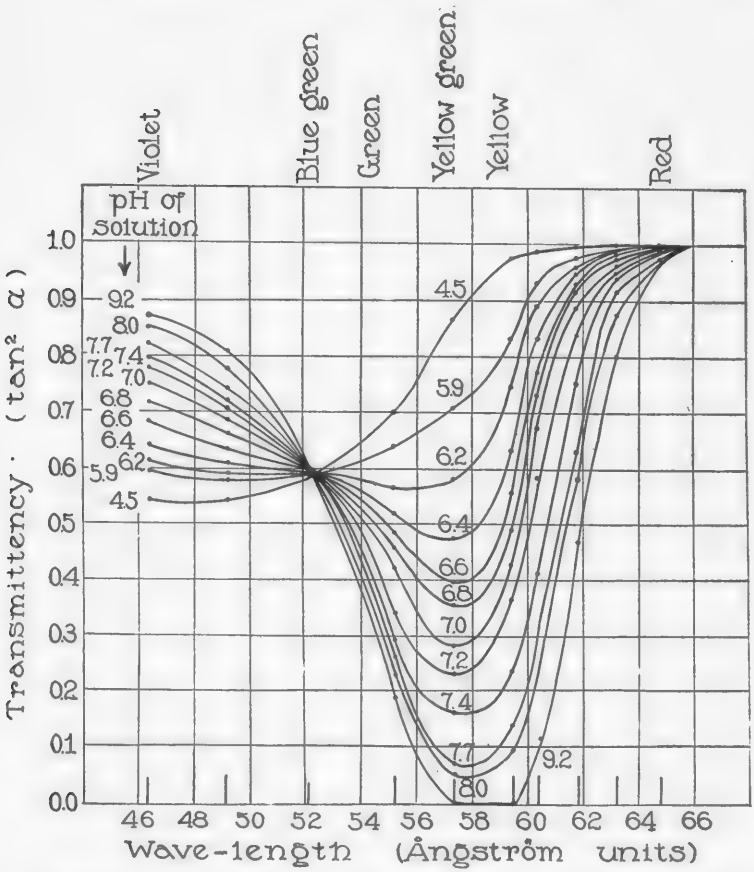
	N/1280	N/640	N/320	N/160	N/80
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
In supernatant liquid	49	56	52	61	58

The concentration of chloride ion in the supernatant liquids was found to be about the same in all. There was proportionately a little more in the gelatin. The determinations were made by adding an excess of silver nitrate to the solutions and titrating the amount of the excess with potassium thiocyanate, with iron alum as the indicator.

The behavior of erythrolitmin, studied in the same way, was found to be completely analogous to that of "whole litmus."

Indicator Properties of Erythrolitmin as Shown by the Spectrophotometer.

The property of certain dyes which enables them to serve as indicators is their ability to absorb different proportions of light in a given part of the spectrum at different hydrogen ion concentrations. The



TEXT-FIG. 1. The absorption of light by 0.01 per cent solutions of erythrolitmin, in buffers, at various hydrogen ion concentrations. The amount of light transmitted by the solutions is plotted along the ordinate, and along the abscissa, in Ångström units, light wave-lengths. Each curve records the variations in light absorption, in various regions of the spectrum, by a single solution of erythrolitmin at a definite pH. The regions of the spectrum at which readings were made are designated on the chart. Within the blue region of the spectrum, the more alkaline the solution, the more blue is transmitted. On the red side of the spectrum, the more acid the solution, the more red comes through.

phenomenon can be readily measured with the spectrophotometer when the instrument has been set to permit the passage of light of known wave-lengths, while allowing light of but one wave-length to pass at any given time. Studies of various dyes have been made in this way, and Brode (18) has recently applied the method to synthetic indicator substances, phenol red, brom cresol blue, and others.

A study of erythrolitmin and azolitmin with the spectrophotometer is of value in that it shows graphically the manner in which the substances act as indicators. It has proved furthermore that they are pure indicators, on a par in this respect with the synthetic dyes examined by Brode.

Four series of Sörensen's phosphate buffer solutions (16) were prepared, each containing 0.02 cc. of 5 per cent azolitmin or erythrolitmin to every 10 cc. As a control solution uncolored buffer was employed. Both were placed in glass cells 10 cm. long. The amount of light absorbed at certain arbitrary points on the spectrum was measured for each solution and a series of curves prepared. Those for erythrolitmin are shown in Text-fig. 1.

The instrument used was of the type first designated by König (19), modified by Martens (20), and made by Franz Schmidt and Haensch (Berlin). The apparatus is briefly described by Sheppard (21). A more detailed description can be found in the paper by Martens and Grünbaum (22), and by Grünbaum (23).

Briefly, the instrument provides for the passage of 2 equal beams of light from a single source, which pass through the solutions to be studied, then through 2 adjustable slits and a collimating lens and are finally refracted by a single flint prism. The emerging beams pass into the movable arm of the instrument which, by a graduated screw, can be placed at any angle thus admitting the light from any desired portion of the spectrum. Here the 2 refracted beams are polarized by a Wollaston calcite prism, passed through a biprism and brought to a focus by a telescope lens.

At the eyepiece 2 images, 1 from each slit, are seen polarized at right angles to each other. By rotating the crossed Nicol prism placed in the eyepiece the intensity of the light in each half of the field is reciprocally altered. Before making any observations the instrument is so adjusted that a movement of the Nicol prism to 45° from the zero point illuminates the 2 fields equally. This was also found to be the case, as it should be, at a rotation of 135°, 225°, and 315°.

Calculation: $\frac{S}{C} = \frac{\mathrm{Sin}^2\, a}{\mathrm{Cos}^2\, a}$

$\frac{S}{C} = \tan^2 a$

Where S = light transmitted through solution containing the dye.

C = light through the control solution.

a = angle of deviation (from 45°).

The amount of light transmitted through the colored solution will vary from 0, when all light is absorbed, to 1, when none is absorbed, that is to say, when the same amount of light comes through on the 2 sides. The light used was a Nernst filament set in series with a resistance coil. The readings of angular deviation were converted to Ångström units, by calibrating the instrument with known spectral lines and interpolating between.

The accompanying figure represents the findings for erythrolitmin. Azolitmin gave practically the same curves. The amount of light transmitted by the solutions is plotted along the ordinate and is simply the reading of $tan^2 a$, subtracted from 1, since $tan^2 a$ represents percentage of absorption. Along the abscissa are plotted the wave-lengths which correspond to the different parts of the spectrum in which the readings are made. Each curve records the variations in absorption of a solution of given pH and it is seen that they fall into a regular order with relation to each other.

It is of great significance that the curves of light absorption in solutions of different pH all cross at a single point, for this is characteristic of pure indicator dyes (18). In this case the point lies at a wavelength of 5250 Ångström units, which corresponds to a blue-green. Regardless of pH the proportion of light transmitted at this point is the same, 58 per cent. On the blue side of the spectrum, the more alkaline the solution, the more blue comes through. On the red side of the spectrum, the more acid the solution, the more red comes through.

The Relative Worth of the Litmus Dyes as Vital Stains.

Rous has recently used litmus as a vital stain in rats and mice (1, 2, 3). For weeks after intraperitoneal injection the tissues of these animals are still colored with it and it has retained its indicator properties. There are numerous differences to be seen which are presumably indicative of local differences in the reaction. We have made an effort in the course of the present work to throw more light on some of the phenomena observed after injection of litmus by comparing with them the effects of injection of the constituent coloring matters.

The litmus used by Rous (1) contained at least the three coloring matters, erythrolitmin, azolitmin, and erythrolein. *A priori* it would seem that the

heterogeneity of litmus might be disregarded when it is employed as a vital stain because of the circumstance recognized by others before us (15) that one of the constituents, erythrolitmin, makes up 90 per cent or more of the whole dye. But many authors have considered azolitmin the active principle of litmus, and conceivably this may have been true of the preparations with which they worked. Our material (Kahlbaum's litmus) which was the same that Rous employed yielded over 90 per cent of erythrolitmin, as already stated. Both azolitmin and erythrolitmin are indicators *in vitro* with practically the same virage and absorption bands, as we have pointed out, but the latter is a more intense dye and, as will be shown below, is more suitable for experiments *in vivo*. The third dye constituent of litmus, erythrolein, is a poor indicator for tissue study since the color change is merely from red to red-orange.

Procedure.

"Whole litmus," erythrolitmin, azolitmin, and erythrolein were separated out of litmus purified as already described, and injected into the peritoneal cavity of rats and mice. The individual dyes like the "whole litmus" were made up in 2 and 5 per cent solutions in 0.9 per cent sodium chloride solution. Sterilization was effected by heating test-tubes partly filled with the solutions in boiling water for 30 to 60 minutes.

The dyes were given with aseptic precautions. Graded doses were administered to many series of animals and special note was made of the rapidity and depth of the tissue staining which ensued. Special cages were employed for the collection of total urine. At various intervals, from half an hour to 10 days after the injections, the animals were put lightly under ether and guillotined. Autopsy was done at once, and the tissues examined. Fresh sections of the kidney, and frequently of other organs, made with the Valentine knife, were studied, and frozen sections were examined later. The kidney findings will form the subject of a succeeding communication. Staining of the tissues was best accomplished by injecting the dyes twice at an interval of 48 hours, killing the animal 2 days after the last injection.

Experimental Findings.

Erythrolein.—No generalized staining followed the administration of this dye. Nearly all of it was soon secreted into the urine which assumed in consequence an intense red color that failed to change notably on the addition of alkali. Moreover, erythrolein proved strongly toxic, causing death in doses of $\frac{1}{2}$ mg. per gm. of body weight.

Azolitmin.—The 5 per cent solution of azolitmin in water or physiological saline proved to be thick and viscid. The indicator was absorbed slowly from the abdominal cavity and much of it could be

found there after 24 hours or more. As a consequence animals were not well colored after the injection of $\frac{1}{4}$ mg. of the dye per gm. of body weight. To obtain satisfactory staining as much as $\frac{1}{2}$ mg. per gm. of animal was necessary, an amount which proved toxic and usually lethal.

Even the largest non-toxic dose stained the kidney but poorly. On the day following a single injection the glomeruli appeared intensely blue from the presence of the dye in the capillary tufts, and blue streaks in the kidney cortex represented the blood vessels in the midst of the pale, unstained parenchyma. The general staining of other organs was slight, but became definite after a second administration of the dye. When examination was made 2 days thereafter, the kidney contained much of the indicator as compared with the amount elsewhere. It was present in the tubule cells for the most part as large red granules though some cells contained blue ones.

Erythrolitmin.—Erythrolitmin has proved far superior to azolitmin or "whole litmus" as a vital stain. *In vitro* it exhibits a greater color intensity than azolitmin, as already demonstrated, and when injected into animals it is much less toxic, doses of $\frac{1}{2}$ mg. per gm. of body weight being well borne. A generalized blue staining with the dissolved dye is caused by but $\frac{1}{4}$ mg. of erythrolitmin per gm. of animal. The injection of this amount causes a pronounced transient bluing of the body. The dye is employed to advantage in 2 per cent solution.

Within an hour after the injection of erythrolitmin into rats and mice blue areas appeared on the pads of the feet and in the ears of the injected animals, and within 2 to 3 hours the entire body was colored blue. After 24 hours little of the indicator could be recovered from the peritoneal cavity, practically all having been absorbed. At this time the animals were diffusely colored and the dye could be found in almost every tissue. Later much of it became segregated in granular form within cells. It is unnecessary to go into the findings in detail since they are identical with those recorded by Rous (1, 2, 3) for rats and mice injected with "whole litmus." After repeated doses the staining became intense and at autopsy most of the tissues appeared highly colored, the kidneys in particular containing large amounts of the dye. Within the tubule cells in

certain regions were granules stained a brilliant red, while in others they were blue, as observed by Rous (1).

The urine voided shortly after the injection was usually red, but, unlike the red urine found after injections with erythrolein, turned blue upon the addition of alkali. But soon excretion of the dye ceased, the urine becoming colorless and remaining so.

"*Whole Litmus.*"—In the course of experiments, to be described in a later paper, numerous rats and mice were injected with "whole litmus" and examined after various intervals of time. The material proved toxic as compared with erythrolitmin and the staining of the tissues effected by it was less intense. The toxic effects would seem from our findings to have been due in considerable part to substances other than the indicator dyes. The various batches of "whole litmus," prepared by the same method, differed greatly in toxicity. Doses of $\frac{1}{2}$ mg. per gm. of body weight of animal were often tolerated well, but at other times proved fatal. The specimens varied much in color intensity as well. Some contained much erythrolein, others little, and differences in the content of azolitmin and erythrolitmin were also found. The first urine collected after injection with "whole litmus" was red, often intensely so, but the addition of alkali caused no change to blue. From the characters of erythrolein, above described, one may conclude that the red color of the urine was due to the excretion of the erythrolein portion of the "whole litmus." Some part of the relative toxicity of the latter may also be ascribed to its content of this pigment.

DISCUSSION.

Caution must be exercised in the interpretation of findings with erythrolitmin and azolitmin when used as vital stains, despite the fact that the light absorption phenomena of these dyes, when prepared as described above, place them on a par as concerns purity with synthetic indicators such as phenol red (18). Both of the dyes are colloidal and they tend to become segregated within the cells in granules. They are dichromatic as well, and because of this may give erroneous information when variously concentrated within cells. The total error from this source and from the presence of salt and protein is smaller, however, than one might expect. And our experi-

ments on the distribution of erythrolitmin and "whole litmus" between a protein and the fluid bathing it, as exemplified by gelatin and water, fail to demonstrate any important influence of the Donnan equilibrium.

While no accurate inferences concerning the absolute pH within living tissues can be drawn through the use of these dyes, changes in the relative reaction can be followed with ease. It is with this in mind that we have studied the indicators.

It is evident from our experiments that one may ascribe the tissue staining described by Rous (1, 2) almost wholly to the erythrolitmin content of the "whole litmus" that he employed.

SUMMARY.

We have devised methods for the separation and isolation of the important indicator constituents of litmus, azolitmin, and erythrolitmin, with a view to employing them as vital stains. Analysis of the color intensities of these dyes shows slight differences in them, azolitmin being the weaker pigment, weight for weight. Study of a third coloring matter, erythrolein, which exists in litmus has shown it to be an unsatisfactory indicator, and toxic for animals.

Analyses with the spectrophotometer of the absorption of light by erythrolitmin and azolitmin, prepared by our methods, and tested over a wide acid-alkali range, show them to be pure substances, comparable in this respect with synthetic indicators.

The errors in the interpretation of the indicator phenomena on vital staining, which are incident to changes in the concentration of the dyes, are so slight as to be negligible. The salt and protein errors on the other hand are large. The factors responsible for the Donnan equilibrium fail to influence the distribution of the indicators between fluid and gelatin.

Erythrolein was found useless when employed for vital staining, and azolitmin proved unsatisfactory since it colors poorly and is toxic. But erythrolitmin can be used to great advantage. It is readily absorbed, and in non-toxic doses stains intensely. The range of pH at which it changes from red to blue fits it for the demonstration of changes in the reaction of living tissues. By reason, however, of the

salt and protein errors to which it is liable, the pH cannot be accurately ascertained.

Intravital staining with erythrolitmin yields results similar to those following injection of purified "whole litmus."

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

XI. The Intracellular Reaction of the Kidney Epithelium and Its Relation to the Reaction of the Urine.

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In the past the injection of dye substances into the circulation of living animals has yielded much information about the functions of certain regions of the kidney (1-7). Recently Rous (8–11) has reported studies of the relative reaction of living cells carried out by the use of various indicator dyes. Some of his methods are well adapted to a study of the reaction within kidney cells and he has observed that litmus when segregated within the renal epithelium is in some places red, in others blue, a fact suggestive of marked functional differences. In the work here to be reported this observation has been followed up and we shall record certain changes in the reaction within cells lining the renal tubules of mammals, which changes accompany and are largely, if not entirely conditioned by alterations in the functional activities of these cells.

Previous Literature.

The older literature on vital staining of the kidney has been reviewed by Cushny (12) and recently by Marshall and Crane (13). The elimination of dye substances by the glomeruli has been demonstrated by Wearn and Richards (14–16) and by Bieter and Hirschfelder (17). Workers are at one concerning it, but there has been much difference of opinion as to the significance of the presence of dye substances within the tubule cells. Heidenhain (1) took their presence to indicate a removal of the dye by these cells out of the blood and into the urine, a secretory activity in other words; but many more recent workers have looked upon it (6, 12) as a result of absorption from the tubule lumen. Recently Marshall and Vickers (18) and Marshall and Crane (13) have reported studies of the excretion of phenol red which they take to indicate a direct secretion of the dye by the tubular epithelium.

Indicator dyes have been much used for the study of kidney function in general but very little for that of the reaction prevailing within renal cells. Long ago Dreser (19, 20) found acid fuchsin in the acid state in the cells of the convoluted and straight tubules of the frog following repeated injections of the dye, and Grützner (21) reported that the glomeruli were stained with indigosulfonate in the acid form under conditions of asphyxia. Rous (22) noted that the color of the renal cortex of rats and mice injected with various of the phthalein indicators was often such as would indicate a pronounced degree of acidity; and more recently Edwards and Marshall (23) have noted that phenolsulfonephthalein when present in the convoluted tubule cells of the dog kidney has the yellow color of acidity. Stieglitz (7) had previously employed azolitmin, neutral red, and sodium alizarinate in a study of changes in the reaction of the renal tissue. He injected the dyes directly into the blood stream of rabbits and dogs and killed the animals within a few minutes. He took the coloration to indicate that the reaction prevailing within the kidney cortex is alkaline when an acid urine is being secreted and *vice versa*. No histological evidence is given in his paper.

In the experiments here to be described we have studied the reaction within kidney cells by means of one of the pigmented ingredients of litmus, erythrolitmin (24–27), which is taken up and held by these cells for considerable periods. Rous, in his papers on the relative reaction of mammalian tissues (8–11, 22) noted that the tubule cells of the rat kidney, after an intraperitoneal injection of litmus, contained abundant granular deposits of the dye, which persisted for weeks and even months after the injection. Since the intracellular material was found on test to have retained its indicator properties it was evident that a study of the reaction within the cells could be made under varying physiological circumstances.

The Suitability of Erythrolitmin for Kidney Cell Studies.

More than 90 per cent of the purified "whole litmus" employed by Rous consisted of erythrolitmin, and it is to this ingredient that the staining he obtained is to be attributed. Erythrolitmin is far superior to azolitmin or to "whole litmus" for purposes of vital staining, being practically non-toxic, and its change from red to blue occurs within the range of hydrogen ion concentration encountered in acid and alkaline urines (12, 24). These facts, coupled with the finding that intraperitoneal injections of the substance did not cause nephritis, as evidenced histologically by sections of the kidney and by the absence of albumin in the urines of the injected animals, led us to employ

it for the present work according to a method described below. Only pure erythrolitmin was used, save where mention to the contrary is made. After intraperitoneal injections the dye is segregated within the tubular epithelial cells everywhere from the glomerulus to the collecting tubule, and shows striking regional differences in color under differing conditions of functional activity. A diffuse staining of the intracellular granules takes place, not a mere surface accumulation of dye such as some authors believe occurs (11) who have dealt with renal epithelium stained in other ways.

Character of the Staining.

The exact situation of dye substances segregated within cells has been the subject of great dispute. The indicator material retained within cells after injections of "whole litmus" is sometimes concentrated upon the surfaces of cell granules and in other cases is diffusely distributed within the latter (8, 9). The colored granules which can be squeezed from renal epithelial cells, after an intraperitoneal injection of erythrolitmin, show in our experience a diffuse distribution of the stain now in dilute form and again greatly concentrated. Many granules spread like masses of a soft gel when pressure is exerted upon the cover slip, but others resist such pressure as can readily be brought to bear and retain their form. There are never any appearances that would suggest a precipitation of the dye. Always it is evenly distributed and the granules are translucent.

Procedure.

Rats of 80 to 200 gm. weight were given intraperitoneal injections of a 2 per cent solution of erythrolitmin in 0.9 per cent sodium chloride solution. The material had been prepared according to a method described in the foregoing paper (24). For every gm. of body weight $\frac{1}{4}$ to $\frac{1}{2}$ mg. of the dye was given and the injection was repeated once or twice at 48 hour intervals. The result was an excellent staining of the kidney. As a rule the organ was examined 48 hours after the last injection, but the findings were much the same when the interval was several hours shorter. At the time for examination of the kidneys the animals were given a whiff of ether, guillotined, stretched out upon a paraffin slab, and submerged in paraffin oil. The kidneys were removed with all possible speed, decapsulated, and sectioned with a Valentine knife while still under the oil. The whole process required often no more than 45 seconds to 1 minute. The sections, protected by oil, were placed on mica slides, covered with thin slips of the same material, and

ringed with paraffin. As these sections were often about 100μ thick, and on the average about 150 to 175μ, observations on the color of the dye granules within the kidney tubule cells could readily be made. Frozen sections also were made and studied to determine the distribution of the dye irrespective of its color. On freezing the tissues, the granules previously diversely colored, red, blue, or violet, became all of one hue, violet, as result of the process of freezing and immersion of the sections in water before mounting. The granules within the cells of fresh sections made with the Valentine knife, on the other hand, did not change color during the first few minutes of observation, often maintaining their original hue for 20 minutes or more, a fact which may be taken to indicate that the coloration was identical with that existing during life.

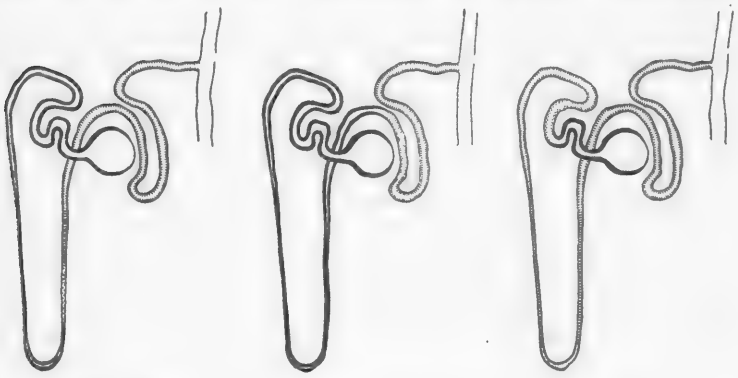
Findings in the Normal Rat Kidney.

In the gross the stained kidneys appeared a dark purple-red. Under the low power of the microscope the outer zone of the cortex appeared blue at first sight, the inner zone red, and the medulla unstained. Closer inspection showed, however, that there were marked local differences underlying the prevailing hues. Much of the renal cortex was unstained. The glomerular tufts showed no litmus but were well filled with blood cells of the natural hue. The endothelial lining cells of the glomerular capsule and particularly those near the entrance of the uriniferous tubule always appeared a deep brilliant blue, containing the dye either in very fine granules, or at times in what seemed to be the diffuse form. Wherever the proximal convoluted tubule could be seen leading off, the cells lining it contained the dye in bright blue granules, which were so abundant as to lend a blue hue to the cells. In most sections this picture was readily to be observed. The glomerular tufts appeared as though in the cup of a blue crescent with a blue stem.

Wherever the eye could follow a single tubule far toward the renal pelvis, the color of the granules contained in the epithelial cells was observed to vary with the region. Near the glomerulus the color was blue, and further down violet, while it was red a very little further on. In that short segment where the tubule cells in general appeared violet, owing to their content of granules having this color, individual epithelial cells were to be found containing granules some of which were brilliant red and others bright blue. In every section there were portions of tubules occupying the zone intermediate between the outer and inner cortical areas of the kidney, that could not be identified definitely. These usually contained violet granules.

In the inner zone of the renal cortex were to be seen in every section many characteristic loops of Henle, mostly of a brilliant blue, but some violet, and a few red. Adjoining these portions bits of tubules were often made out from just above or just below the loops of Henle in which a transition from blue through violet to red or *vice versa* could be seen. The above mentioned unidentifiable bits of tubules containing the dye in violet form may be considered to have come from regions lying a little way above or below the loops of Henle.

In the outer cortical region convoluted tubules could be seen the cells of which all contained the dye in red granules. In no instances were the striations, characteristic of proximal convoluted tubules, present among them. Very often these "red tubules" emptied directly into the collecting tubes, in which case there was, of course, no doubt that we were dealing with distal convoluted tubules. In striking contrast, the proximal portions of the convoluted tubules, when definitely identified, either by their striations or when emerging from a glomerulus, contained the dye in the blue form. Never was any dye to be seen in the cells of the



TEXT-FIG. 1. TEXT-FIG. 2. TEXT-FIG. 3.

Text-fig. 1 shows diagrammatically the renal color pattern following intraperitoneal injections of erythrolitmin in rats secreting neutral urine. Text-figs. 2 and 3 show the changes in color pattern associated with the secretion of alkaline and acid urine, respectively, described in the text. The region of blue erythrolitmin is indicated by solid black, that of violet by dots, and of red by the short lines.

collecting tubules and none was ever recognizable in the glomeruli or tubular lumena.

Sometimes all of the findings described could be made out in a single preparation. From the examination of scores of fresh sections, from many animals, we have prepared Text-fig. 1, which shows diagrammatically the state of affairs in the kidney of a rat maintained on an ordinary diet of barley, oats, and bread and milk, and excreting a very slightly acid or a neutral urine.

In brief, then, during the excretion of neutral or very slightly acid urine, the granules of the lining cells of the glomeruli as well as those

of the cells of the derivative tubules, at times as far along as the loops of Henle, are alkaline to erythrolitmin. Further on, in a short segment of tubule the cell granules show the violet color of neutrality, while further yet, in the cells in the ascending limb and in the distal convoluted tubules, the granules are acid in reaction (Text-fig. 1). There is no dye anywhere to be seen in the lumina of the tubules and none within the cells of the collecting system.

The Effect of Diuresis on the Staining with Erythrolitmin.

A number of rats were provided for several days with nothing but a 10 per cent watery solution of cane sugar which they drank in great quantities. During this period erythrolitmin injections were given as in the instances described above. A series of control animals fed on a diet of bread and milk, barley and oats and injected with similar doses of erythrolitmin, were employed for comparison. The urine secreted by the sugar-fed animals appeared slightly more alkaline to brom cresol purple and phenol red than did that of the controls and was from two to four times as abundant. In the kidneys of individuals killed at the height of the diuresis, sections of the renal tissue, viewed in the gross, appeared to contain far more blue dye. Microscopically, fewer unstained areas were seen and the loops of Henle were oftener of a bright blue, though the cells of the distal convoluted tubules were still always red. The point of change from the blue to the red reaction was apparently situated further down the tubule.

The experiments suggested that with an increase in the quantity of the urine and a change to slightly greater alkalinity, more, relatively speaking, of the litmus was present in the alkaline form.

The Kidneys during the Excretion of Alkaline Urine.

Animals to which sodium bicarbonate had been given were now studied.

Rats weighing from 75 to 200 gm. were given by stomach tube, in the morning and again late in the afternoon of the same day, 5 cc. of a 10 per cent solution of sodium bicarbonate. A plentiful excretion of alkaline urine always resulted. On the 2 or 3 days following, during which the bicarbonate administration was continued, injections of a 2 per cent erythrolitmin solution were made as in the

normal control rats. There were either two or three injections, on successive or alternate days. After a final lapse of 48 to 72 hours, without discontinuance of the bicarbonate administration, the animals were killed and the kidneys examined microscopically. To vary the conditions some individuals, previously injected twice or thrice with erythrolitmin, were given the alkali once, then again on the following morning, and in the afternoon they were killed and studies of the kidneys were made. The findings were the same whichever the procedure.

In the gross the kidneys appeared deep purple-red as ordinarily. Microscopically the general color of the cortex was blue, but scattered areas of cells with brilliant red granules were to be found. As usual the glomeruli lay within blue crescents of endothelial cells packed with blue dye, and the capillary tufts were filled with blood of natural color. The cells of the proximal convoluted tubules leading away from the glomeruli were crowded with granules of a dense deep blue. Nearly all the tubular loops which could be identified as descending limbs of the loops of Henle were deep blue and so too with the ascending limbs for some distance. In the cortex, however, many red tubules were seen, the cells thereof containing in no instance the striations of proximal convoluted tubules. A connection of these red tubules with the collecting tubes could frequently be made out and hence they must have been portions of the distal convoluted tubules. But segments of them,—not adjacent to the collecting tubes but lying further up,—were blue or violet, a phenomenon never observed in the animals secreting neutral urine. The collecting tubules themselves were unstained.

A change in the relative reaction within the tubule cells as evidenced by the distribution of the litmus colors had evidently taken place in these animals. The blue coloration extended much further down, being seen in the granules of the cells of the ascending limb of the loop of Henle and in an adjoining portion of the distal convoluted tubules. In every case the terminal portions of these latter where they joined to the collecting tubules were red with the indicator, as under normal conditions, and this despite the fact that a markedly alkaline urine was being secreted.

The findings described above have been gathered from an examination of many sections from many animals, though occasionally a single specimen showed them all. By and large they demonstrate clearly that, during the secretion of alkaline urine, the reaction within the cells of the kidney cortex is markedly altered. The essential difference from the normal lies in an extension of the blue color along the tubular path, in some instances almost as far as the collecting tubules. Text-fig. 2 shows this diagrammatically.

Appearance of the Kidneys during the Secretion of Acid Urine.

Rats injected with erythrolitmin were caused to secrete acid urine. The kidneys showed changes the opposite of those occurring when the urine had been rendered alkaline.

The secretion of acid urine was brought about in rats by the administration of a 10 per cent aqueous solution of sodium acid phosphate through a stomach tube. At the same time control animals were fed ordinary food and other rats were fed alkali for the experiment already described. Rats of like age and weight were chosen for the procedures. Half of those given sodium acid phosphate received two doses in a single day and 24 hours later the first of the injections of erythrolitmin. Either two or three injections of the dye were made, on alternate or successive days, and the examination of the kidneys was carried out 48 hours after the last injection. Throughout the period of preparation, 5 to 8 days in all, the feedings of sodium acid phosphate were continued; and the animals' urine was regularly acid during the period. The other half of the rats were fed acid solution for the first time on the day after the last of several erythrolitmin injections, and again on the following morning. 6 hours later the kidney examination was made, at a time when acid urine was being formed. In both groups the findings were the same.

In the gross the kidneys appeared reddish purple. Microscopically the sections appeared almost entirely a bright red in contrast to the spotty red and blue of the controls. The outer areas of the cortex were red, with only here and there tubules containing the dye in blue form. The tubules of the inner cortical area were all red, including the loops of Henle. But it was striking that even in these kidneys the glomeruli lay cupped in blue crescents just as in the controls, the endothelial lining cells containing erythrolitmin only in the blue form. Wherever a tubule could be found leading off from the tuft the cells near the glomerulus likewise contained blue granules. Text-fig. 3 shows diagrammatically the findings in these experiments. The cells of the first portions of the proximal convoluted tubules contained the dye in the blue form only, but the changes from blue to violet and then to red occurred close to the glomerulus, considerably nearer than in normal animals, and far nearer than in the kidneys of the rats fed alkali. Frequently the shift in color of the intracellular granules was seen to occur at a point nor more than 100 or 200 μ from the exit of the tubule from the tuft. In numerous instances the eye could trace the entire color sequence within a relatively short extent of tubule. Many individual cells in the region of color change where the granules were preponderantly violet contained both bright blue and brilliant red granules lying together in the cytoplasm. Distal to the region of change all of the intracellular granules were red. The few blue areas in the cortex could always be identified as segments of the proximal convoluted tubules from next the glomerulus. Cells containing dye in the blue form were never found further along. Brilliant

red areas were frequent that consisted of proximal convoluted tubules. No blue loops of Henle were seen; all were red. As in the other sections the collecting tubules were quite without color.

To sum up the alteration in the reaction within the tubule cells, as evidenced by the litmus change to red, affected the proximal convoluted tubules throughout almost their whole length and the entire loop of Henle. The structures mentioned now showed acidity instead of the alkalinity which is normal to them.

Constancy of the Findings.

The phenomena described occurred with but very little individual variation. Certain of them were constant. No matter what the reaction of the urine the granules of the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules were blue. And the intracellular granules of the further portion of the distal convoluted tubules were always red. Erythrolitmin was stored throughout the system from glomerular tuft to collecting tubule but not in either of these structures. In normal animals secreting a neutral or slightly acid urine the transition from red granules to blue, the zone that is to say where the granules are predominantly violet, is situated in the beginning of the ascending limb of the loop of Henle.

The relative reaction of kidney cells and their changes under functional conditions were studied in some other species besides the rat. Mice, guinea pigs, and rabbits were given intraperitoneal injections of erythrolitmin in dosages similar to those used in the rat experiments, that is to say $\frac{1}{4}$ to $\frac{1}{2}$ mg. of the dye per gm. of body weight. The secretion of acid and alkaline urines was induced in the way already described, by feedings of sodium acid phosphate or sodium bicarbonate; and the state of the kidneys was compared with that of normal animals injected with erythrolitmin. The findings were altogether similar to those in the rat, so they need not be enlarged upon.

Constancy of the Findings Following Massive Doses of Acid and Alkali.

Acid Administration.—A remarkable feature in the experiments just described was the regular occurrence of erythrolitmin in the blue form

TABLE I.

Rat No.	Body weight	Daily administrations of HCl (concentration and amount)	Character of secretion of urine	Urine pH	Interval between last acid dosage and autopsy	Color of erythrolitmin in glomerular lining cells		
	gm.				*hrs.*			
1	142	1 of N/10, 10 cc.	Copious secretion	6.0	16	Blue		None red
2	150	4 of N/10 @ 10 cc.	Copius secretion	5.6	16	Blue		None red
3	122	2 of N/10 @ 10 cc.	Few drops only on 2nd day	6.0	5	Blue	Some blue-purple	None red
4	128	1 of N/6, 5 cc.	Copious secretion 6 hrs .later	5.9	6	Blue	Some blue-purple	None red
5	125	1 of N/3, 5 cc., followed by water, 9 cc., in 1 hr.	Scanty secretion 2 hrs. later	5.4	2	Many blue	Many purple	None red
6	135	1 of N/4, 9 cc.	Scanty secretion 6 hrs. later	5.5	6	Many blue	Many purple	None red
7	99	1 of N/4, 10 cc.	Very scanty secretion 5 hrs. later	5.9	5	Many blue	Few purple	None red
8	104	" "		to		Change from blue to red in proximal convoluted tubules very close to glomerulus		
9	111	" "		6.2				
10	106	1 of N/4, 10 cc.	Hematuria 2 hrs. later. Suppression 5 hrs. later		5	Very few bluish purple	Mostly purple	A few red
11	104	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5 to 6.0	5	Few blue	Many purple	None red
						In proximal convoluted tubules cells near glomerulus purple to red		
12	129	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5	5	Many purple		Red in two glomeruli

13	120	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.0	5	None really blue		Several red-lavender
14	110	3 of N/10 @ 10 cc. 1 of N/2, 2 cc.	Last day suppression		16	None blue		Many red
15	154	3 of N/10 @ 10 cc., a 2 day interval, then 1 of N/2, 2 cc., then 1 of N/4, 10 cc.	Last day suppression		7	None blue	Many purple	Some red
16	101	1 of N/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 6 hrs later suppression		6	Few blue	Mostly purple	Some red
17	109	1 of N/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later suppression 4 hrs. later suppression		4	None blue	All lavender	None red
18	122	1 of N/2, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 5 hrs. later suppression		5	Few blue	Mostly purple	Some red
19	168	1 of N/1, 14 cc.	1½ hrs. later hematuria 3 hrs. later suppression		3	Few blue	Mostly purple	A few red

within the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules, no matter what the reaction of the urine that was being secreted. Litmus segregated within healthy cells elsewhere throughout the body is regularly in the acid form, red that is to say. It was deemed worth while to test whether the blue of the glomerular litmus is changed to red by massive doses of acid.

For the purpose, rats weighing from 99 to 168 gm. were given dilute HCl by stomach tube in varying concentrations and amounts, as outlined in Table I. As a rule, several administrations of HCl were employed at 24 hour intervals, or a single dose in higher concentration. The animals were kept in special cages for the collection of urine under oil. Fresh bladder urine unmixed with feces was obtained for pH estimation with indicators by holding a closed cone over the animal's nose for a moment, thus inducing a struggle during which urine was voided. In all other respects the technique was that already described.

Table I summarizes the result of this experiment. The animals receiving the smaller quantities of acid (Nos. 1 to 9, inclusive) continued to secrete urine. In the kidneys of these rats erythrolitmin, present in the lining cells of the glomerulus and epithelial cells of the first portion of the proximal convoluted tubules, was still always blue. In both of these regions it frequently appeared bluish purple, but never frankly red. The color change of the dye in the cells of the proximal convoluted tubules took place much closer to the glomeruli than in the earlier experiments, often less than the distance of one glomerular diameter away from the exit of the tubule from the tuft.

Suppression of urine, usually preceded by hematuria, followed the large quantities of acid given to Animals 10 to 19 , inclusive (Table I). In contrast to the findings of the foregoing experiments, erythrolitmin appeared at times in the red form within the glomerular lining cells and those of the first portion of the proximal convoluted tubules. But only after urinary suppression could this be demonstrated. Whenever secretion continued (Rats 1 to 9 inclusive), the dye in the locations named remained either blue or a deep bluish purple. A comparison of the findings of Rats 7, 8, and 9 with those of 10 shows this point well, for all these animals received similar amounts of acid but with differing results. So, too, does a comparison of the findings of 3 with those of 11, 12, and 13.

The effect of massive doses of acid may also be evoked by means of an "acid-forming" diet. Feedings of $CaCl_2$ have been shown by Haldane, Hill, and Luck (28) to have the effect of administrations of HCl; for the calcium moiety is not retained, while the chlorine is. According to Gamble, Ross, and Tisdall (29), 40 per cent of the chlorine given in this way behaves in the body like HCl. As Addis, MacKay, and MacKay have pointed out (30), a rat eating 10 gm. of diet containing 4 per cent of $CaCl_2$ should receive the equivalent of 14.4 cc. N/10 HCl.

We therefore maintained rats for 10 days on a diet of bread and milk to each 100 gm. of which 4 to 6 gm. of $CaCl_2$ were added. Eight of the animals were

injected with erythrolitmin on 2 alternate days and killed 48 hours after the last injection. The special diet was continued to the end. Urine was secreted plentifully and of a pH usually well below 6.0. Upon examination of the kidneys the dye still appeared blue in the glomerular lining cells and the first portion of the proximal convoluted tubules.

Alkali Administration.—Massive doses of alkali were given to rats stained with erythrolitmin, to see whether in this way the color of the dye present in the lining cells of the final portions of the distal convoluted tubules could be influenced. Even after the feedings of sodium bicarbonate outlined earlier in this paper it had been constantly in the red form.

Stained rats were given by stomach tube 4 cc. or 2 cc. of N/1 Na_2CO_3 solution per 100 gm. of body weight. The larger doses led to rapid suppression of urine, the smaller often to a similar result after 48 hours. The urine when obtained was usually of a pH about 8.2, as determined by the indicators cresol red and thymol blue. Examinations of the kidney were made, in many instances, before this result had been attained, and in others afterwards. We were never at any time able to demonstrate the dye in the blue form within the lining cells of the final portion of the distal convoluted tubules where these were actually observed emptying into the collecting tubules. The dye was frequently of a reddish lavender but never frankly blue. In all other situations throughout the tubular epithelium, it was intensely blue.

It is evident that massive doses of acid fail to change from blue to red the erythrolitmin segregated in the cells lining the glomeruli and the first portion of the proximal convoluted tubules. As long as the kidney continues to secrete urine these cells maintain a relative alkalinity, despite the strong acidity of the urine. Only in the event of urinary suppression is there some slight shift in the direction of acidity.

After massive doses of alkali, sufficient sometimes to cause urinary suppression, the erythrolitmin within the cells lining the final portion of the distal convoluted tubules remains red.

Results after Intravenous Injections.

To complete our observations some intravenous injections of erythrolitmin were made. The method has already been used by Stieglitz (7) who employed azolitmin, neutral red, and sodium alizarin upon rabbits and dogs.

Rats of approximately 100 gm. weight were injected intravenously with 1 or 2 cc. of a 1 per cent solution of erythrolitmin at a pH of approximately 7.0. The kidneys were extirpated and sectioned after intervals of 30 minutes, 1 hour, 2, 5, 6, 10, and 18 hours. The animals tolerated the dye poorly and in many instances became moribund a few minutes after the injection.

The findings were by no means as clear-cut as after intraperitoneal injection of the indicator since the kidneys never became well stained.

Within a few seconds after intravenous injection of erythrolitmin the urine contained it in high concentration whereas the kidney tissue showed not the slightest traces. When half an hour had elapsed the glomeruli appeared faintly bluish regardless of the reaction of the urine. The color seemed to be due to the presence of the dye in the blood within the tufts. When 2 hours had elapsed some of the tubular epithelial cells of kidney sections showed a very faint diffuse blue tint. No definite coloration of intracellular granules could be made out, and the staining was so slight that it appeared merely as a blue haze over the cells. 6 to 8 hours after an injection of the dye, colored granules could be found here and there in the tubule cells. Both red and blue granules were to be found, indiscriminately located. In this connection it may be recalled that litmus is often taken up by cells in fine blue particles and only secondarily converted to red (9) as further that when a cell containing segregated litmus is damaged or has begun to degenerate it turns from red through violet to blue (9). The coloration of the kidney cells was so poor, as compared with that obtained after intraperitoneal injections, that no conclusions as concern kidney function could be drawn from the instances mentioned.

With a further increase to 24 or 48 hours in the time interval between the injection of erythrolitmin and the examination of the kidney, the appearance of the organ came to resemble closely that following intraperitoneal injections. Nothing, then, had been gained by the intravenous administration of the indicator.

We were unable to secure satisfactory staining of the rat's kidney after intravenous injections of azolitmin (Grübler), with our own preparation of azolitmin or with sodium alizarin VI, dyes with which Stieglitz (7) reported success in dogs. Exitus frequently occurred soon after injection of the dyes when given in quantities too small to stain the kidneys.

It has been reported by Stieglitz that, after the intravenous injection of azolitmin, sodium alizarin, and neutral red, the reaction of the kidney cortex is apparently the opposite of that of the urine secreted at the time. In view of our failure to confirm this finding with the aid of the dyes just mentioned it seemed necessary to repeat Stieglitz's experiments with neutral red.

Solutions of neutral red in physiologic saline when given intravenously stained the kidney well in dogs and rabbits within a few minutes. The tubule cells took up the dye in both a diffuse and granular form and it appeared promptly in the urine. However, under these circumstances there was no marked differential staining, as with erythrolitmin. The kidney was always of an almost uniform hue, which hue depended largely, as will be shown, upon the dosage of the dye and the time interval between its injection and the examination of the organ. In passing it should be said that the yellow, alkaline form of neutral red can easily be confused with the natural yellowish color of thin sections of kidney tissue.

Six dogs and 16 rabbits were induced to secrete alkaline or acid urine, as desired, through the oral administration of sodium bicarbonate or sodium acid phosphate, or by the intravenous injection of these substances. After the bladder urine obtained by catheterization had become definitely alkaline or acid, intravenous injections of neutral red (Grübler) were given, in 1 per cent or 0.5 per cent solution in physiological saline. Various doses were used and the kidney examined after various intervals of time. Specimen protocols follow:

Normal dog, 8½ kilos.

10. 50 a.m. Ether anesthesia induced; the bladder urine is distinctly acid to brom cresol purple and neutral red. 10.52. Catheterization; urine specimen acid as before; catheter bound in.

10.55. *30 cc. of 1 per cent solution of neutral red (Grübler) in 0.9 per cent NaCl solution injected intravenously.* The animal became rose red at once. 11.01. The dye appeared in the urine, in the red form. 11.25. The right kidney was removed through a posterior incision. In the gross it appeared deep red. In sections, cut with a Valentine knife and mounted between mica slides, the tubular epithelial cells were colored red, and many contained the dye in granular form. The general staining of the kidney, however, was slight.

11.30. *Intravenous injection of 5 per cent solution of sodium bicarbonate, 150 cc.* 11.33. The urine became orange. 11.40. The urine was clear and dark yellow; it turned red upon the addition of acid. 12.00 m. The left kidney was removed. In the gross the whole organ appeared yellowish. Sections, made with the Valentine knife and mounted as described above, appeared yellow. The tubular epithelial cells were stained diffusely yellow.

In this experiment the kidney removed after the animal had begun to secrete alkaline urine appeared definitely more alkaline than the organ examined at a time when the urine was acid.

In another experiment after an intravenous injection of 5 per cent sodium bicarbonate with consequent elimination of alkaline urine, a larger intravenous injection of 1 per cent neutral red was given, 45 cc. to a 9 kilo dog, and the kidneys were removed 12 minutes later instead of after half an hour, as in the experiment above. Microscopically the epithelium of the renal tubules appeared red although the urine secreted was yellow, a finding the opposite of that just described, but in agreement with the experiments of Stieglitz (7). The test was repeated on

another 9 kilo animal with the same dosage of dye but one of the kidneys was removed half an hour after an alkaline reaction of the urine had been established, instead of after the lapse of but 12 minutes as in the previous experiment. The second kidney was removed 30 minutes after an acid reaction of the urine had been established. With this greater interval of time between the injection of dye and the examination the color of the kidney cortex was found similar to that of the urine secreted. The protocol follows:

Normal dog, 9 kilos. All urine specimens obtained by catheter.

10.45. a.m. Ether anesthesia. 10.50. Urine clear and strongly acid to neutral red, erythrolitmin, and brom cresol purple.

10.58. *Intravenous injection of sodium bicarbonate, 5 per cent solution, 75 cc.* 11.08. Intravenous injection of neutral red, 1 per cent, 40 cc. 11.14 (6 minutes later). Urine appearing red from the catheter,—still acid. Intravenous injection of sodium bicarbonate repeated. 11.17. Urine bright orange, that is to say definitely alkaline. 11.47. Urine has been alkaline for ½ hour. Right kidney removed. Histological study made at once (see below).

11.49. *Intravenous injection of sodium acid phosphate solution, 5 per cent, 70 cc.* 11.51. Urine now reddish rose colored. 11.53. Injection of sodium acid phosphate repeated. 11.55. Urine bright rose red, strongly acid. 12.21 p.m. Urine has been acid for ½ hour; left kidney removed. Histological study made at once. Animal killed with chloroform.

The kidneys were removed, in each instance 30 minutes after the urine had been rendered acid or alkaline, respectively. When the animal was passing yellow alkaline urine the gross appearance of the kidney was a reddish yellow. Microscopically, sections mounted on mica slides and ringed with paraffin showed a moderate, diffuse, and granular staining of the cortex. The dye in the tubule cells was mostly in the yellow form, but some in the red. The predominating color, though, was yellow, like that of the urine.

When acid urine was being secreted the kidney appeared bright red in the gross. Nearly all the dye in the epithelium of the tubules was present in the red form, with here and there a little yellow coloration. Again the predominating color of the cortex was like that of the urine.

Such experiments were many times repeated, with different doses of neutral red. The dye was given in the red form, but was brought close to the neutral point with sodium bicarbonate. It cannot be injected in the yellow form because of the resulting precipitation of the dye in alkaline reaction. In general, when large doses of the dye were given and examination of the kidney made shortly afterwards, the tubule cells contained much of it in the red form, regardless of the reaction of the urine. When half an hour or more was allowed to elapse between the injection of the dye and examination of the kidney,

and when the dosage of dye was not too great, the majority of epithelial cells manifested the same color as did the urine.

Similar experiments upon rabbits gave findings of like sort. When large doses of neutral red were employed and the examination made soon thereafter, the kidney cortex, in general, appeared red, no matter what the reaction of the urine. Lengthening the interval between injections of the dye and examinations of the kidney gave time for the organ to convert most of it in the tissues to the form in which it appeared in the urine.

The protocol which follows contains the findings in four rabbits, and shows well the influence of dosage, and time interval, upon the coloration of the kidney cells with neutral red.

The treatment of the animals may be tabulated as follows:

Rabbit	*a*	*b*	*c*	*d*
Weight	650 gm.	655 gm.	647 gm.	653 gm.
Intravenous injection of 5 per cent sodium bicarbonate	8 cc.	8 cc.	8 cc.	8 cc.
5 min. later. Intravenous injection of 1 per cent neutral red	12 cc.	2 cc.	12 cc.	2 cc.
Kidney examination	5 min. later	5 min. later	90 min. later	90 min. later

After the bicarbonate injection the urine of all the animals was strongly alkaline and contained much dye in the yellow form.

Rabbit a.—In the gross the kidney appeared dark red. Section showed the tubule cells containing much red dye, in granular as well as diffuse form. The cortex was predominately red.

Rabbit b.—The kidney appeared red in the gross, but lighter in color than that of Rabbit *a*. Sections were yellowish red. The tubule cells contained dye in red granules. The general appearance of the kidney of *a* was more acid than that of *b*. N/50 HCl run under the cover-glasses turned the sections as a whole a deeper red, showing that some of the dye was present in the yellow form.

Rabbit c.—The kidney appeared a mottled reddish yellow. The sections were red in general and deeply stained. Many tubule cells held the dye in red granular form, while in other tubules the cells contained yellow granules. The glomeruli appeared as distinct yellow crescents. The urine was a deep yellow. The

bladder, however, contained yellow urine with clot-like masses of dye in the red form. When the contents were mixed the prevailing hue was yellow.

Rabbit d.—In the gross the kidney appeared yellow. Sections were also yellow. There was more dye present in the tubule cells than in those of Rabbit *b* which received the same dose of the indicator, and less than in Rabbits *a* and *c*. Practically all of it was present in the alkaline form.

From the results of these and other like experiments certain facts became plain. When the dose of neutral red was small the tubular epithelium of the kidney usually appeared of the same color as the urine secreted, though in a few instances it had the opposite color. When the dose was large enough to stain the tissue well much of the indicator within the cells manifested the color it had when injected, regardless of the reaction of the urine. When the time interval between the injection and the examination of the kidney was short, that is to say half an hour or less, the cells might or might not have the same color as the urine. If the time interval was lengthened, the color of the majority of the kidney cells was that of the urine being secreted. Apparently the lengthening of the interval between the intravenous injection of a large amount of dye and the histological examination gave time for its conversion in the stained tissues to the form in which it appeared in the urine. If the time interval after injection was short, it seemed largely a matter of chance how the kidney was stained owing to the great concentration and overwhelming amount of material suddenly brought to it.

Altogether it is obvious that the results of the intravenous method of administration of indicator dyes for renal staining are untrustworthy, unless examination of the organ can be postponed until the cells can deal with the material coming so suddenly to them. In practice many indicator dyes are toxic, when administered in this way, and the animals frequently die before sufficient time for a proper staining of the tissues has elapsed.

DISCUSSION.

Erythrolitmin is far from being a perfect indicator (14). In view of this fact how much is one justified in inferring from the present work? The color of most indicators varies with their concentration

and erythrolitmin is no exception to the rule. There is then a definite, though slight, increase in the red in higher concentrations; and since erythrolitmin would appear to be often very highly concentrated when segregated within the kidney tubule cells, the possibility of an interpretative error from this source may be great. The dye is subject to large salt and protein errors as well, but these influence it in the opposite direction. Erythrolitmin in the presence of salt or protein at a given pH appears more blue than in solutions at the same pH without these ingredients (24).

Granting these facts erythrolitmin still has notable virtues as an indicator of tissue conditions. It persists within the tissues for months, eliciting no evident reaction and retaining its indicator characteristics. This is true of the dye segregated within renal epithelium. When a section of the stained kidney is placed in a buffer solution all the stained granules take the same color, namely one approximating that which would be expected of erythrolitmin at the pH of the buffer. In untreated specimens immediately after removal from the body many granules containing the dye in highly concentrated form are a deep blue. Since the optical error upon concentration is toward the red, it is evident that this factor plays no decisive part in the color.

Can the findings of the experiments outlined in the present paper be due to supravital changes? The constancy of the results under a given set of conditions and the equal constancy of the induced variations would seem to put this possibility out of the question. One might suppose that the knife in sweeping from cortex to medulla and carrying with it a mixture of blood, lymph, glomerular filtrate, and more or less completed urine would bring about an alteration in the granules of the cells with which it came in contact. So it must in those cells that are injured. Such an effect will not explain, however, the orderly arrangement of red and blue granules everywhere in the unexposed cells. These cells show no change in the litmus coloration for many minutes after removal from the body. The kidneys can be removed from living animals, sectioned, and observed, within 45 seconds to 1 minute. Renal cells can scarcely be regarded as dead in so short a time, much longer interruptions of the circulation being withstood (31).

Can one suppose the variations in the reaction of the granules in the living cells of the renal tubules to be a mere passive reflection of the reaction of the fluid passing along the tubules? Such a view is incompatible with what is known of cell integrity. Litmus segregated in granular form by phagocytes does not take the color appropriate to the hydrogen ion concentration of the fluid surrounding these cells unless they are crushed or otherwise injured (9).

All things considered, one is forced to the conclusion that the color patterns seen in kidneys stained with erythrolitmin are the expression of functional activities. But how these activities exert their influence on the reaction of intracellular granules is a problem of great complexity.

The fact has already been emphasized that the granules within the epithelium of the glomerulus and the adjoining portion of the convoluted tubules are regularly blue, no matter what the reaction of the urine. Were it not for the cell integrity just mentioned the finding might seem explicable on the basis that the renal filtrate as derived from the blood and bathing these cells is ordinarily alkaline. Everywhere else though throughout the body litmus-containing elements are bathed by alkaline fluids yet the color of the indicator within them is red, indicative of acidity. Why should there be this peculiar exception in the case of this region of the kidney? And what is one to think of the shifting in the renal color pattern that can be brought about by administering substances to the animal which cause the reaction of the urine to shift to the alkaline or acid side. The tubule cells adjoining Henle's loop constitute, as it were, a transitional zone of color change. In them, erythrolitmin granules are violet, under normal circumstances, or sometimes red and blue in the same cell. During the secretion of acid urine the cells of this region contain the dye in the red form. One might suppose the red of the granules in this region to be of no greater significance than that met elsewhere in the body were it not for the fact that the red can be altered to blue by altering the functional conditions, as during the secretion of alkaline urine. One can only suppose these phenomena to be an indirect expression of functional activities on the part of the renal cells—cells through which material is passing into or perhaps out of the blood. Only in the case of the lining of the distal convoluted tubules where these join the collecting

system is another explanation possible. Through thick and thin, so to speak, the litmus within such cells remains in the red form, the form in which it is encountered in healthy, non-secreting cells elsewhere in the body. Here there would seem to be no reason to invoke a functional passage of material through the cytoplasm in order to explain the findings. Since the reaction within these cells cannot be rendered alkaline by any of the experimental means as yet attempted, it is conceivable that it is held fixed, as is certainly not the case higher up. Yet since these cells differ in no wise morphologically from those immediately adjacent to them in the same tubule, and do differ sharply from the elements of the collecting tubules upon which they abut, it seems more likely that they are protected by a fortunate situation from those functional stresses which in our experiments led to a change in the reaction of the granules further up.

Granted that the renal color pattern and its changes are the expression of functional activities, what is the nature of these latter? Do the cells lining the tubules resorb fluid only, or secrete materials into the tubular lumen as well? Unfortunately the findings here described would appear to admit of both possibilities. One can say at the moment only that the reaction within the cells of the renal tubules varies from region to region in a way which shows it to be intimately connected with the secretory activities of the organ. In some regions it is profoundly altered by changes in these activities.

Stieglitz' (7) conclusion that the reaction of the kidney cortex is the reverse of that of the urine being secreted at the time is based on observations of the cortex color following intravenous injections of indicators. Our repetition of some of his experiments has shown that the color of the kidney cortex shortly after such injections is influenced chiefly by factors not considered by Stieglitz, namely the dosage of the dye and the variations in the time interval between its injection and the examination of the organ. Large amounts of indicator dye coming suddenly to the kidney from the blood may be found, a few minutes later, unchanged in color within the cortex cells regardless of the reaction of the urine. The kidney overwhelmed, so to speak, with dye requires an appreciable time to act upon all the indicator substance present. A heavy dose of neutral red, in the acid form, injected intravenously into animals secreting alkaline urine, will appear in the

kidney cortex a few minutes later still in its red, acid form, indicating a reaction the reverse of that of the urine being secreted at the time. Later the dye in the cortex will be in the yellow, alkaline form. Small doses of neutral red intravenously injected in the acid form into animals secreting alkaline urine are of course converted to the alkaline form in the kidney sooner than are large doses. When acid urine is being secreted, neutral red injected in the acid form remains unchanged regardless of the dosage given.

Such findings clearly show how the employment of indicators by intravenous injection may lead to erroneous conclusions concerning the relation between the reaction of the kidney cortex and that of the urine secreted. They show further that the examination should be postponed until the cortex cells have had ample time to deal with the dye coming to them. Under these conditions one finds the reaction of the kidney cortex to be, by and large, the same as that of the urine under secretion.

SUMMARY.

The tubules of the mammalian kidney, vitally stained with erythrolitmin, show a significant color pattern, the cells of certain regions appearing bright blue and others brilliant red. The dye is segregated within the cytoplasm, staining fine granules diffusely.

Under normal circumstances of renal function erythrolitmin is stored in the lining cells of the glomerulus and the epithelial cells of the proximal convoluted tubules in the blue, alkaline form. In the cells of the final portion of the distal convoluted tubules the dye is red. Between these regions a narrow transitional zone can be found, at times above, at times below the loop of Henle, in which the erythrolitmin-stained granules in the tubule cells are violet, or red and blue within the same cell.

Alterations in the relative reaction of certain regions of the tubules, as disclosed by the color of the dye within the cells, can be induced by means that alter the reaction of the urine. When acid urine is being secreted there is a change from relative alkalinity to acidity high up in the tubular canal, in the proximal convoluted tubules. During the secretion of markedly alkaline urine the intracellular granules appear blue most of the way down the tubular canal, even to the first portion

of the distal convoluted tubules. When the urine is neutral the cells above the descending limb of the loop of Henle are alkaline to erythrolitmin, and those below this point appear acid.

The granules within the endothelial cells of the glomerulus and the epithelial cells of the first portion of the proximal convoluted tubules are always alkaline to erythrolitmin; while those within the cells of the final portion of the distal convoluted tubules are always acid regardless of the reaction of the urine. Only after complete suppression of urine as result of massive doses of acid do granules of the sort first mentioned manifest the color indicative of acidity.

The interpretation of the findings waits upon further work.

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ETIOLOGY OF OROYA FEVER.

XII. Influence of Malarial Infection (Plasmodium Inui?), Splenectomy, or Both, upon Experimental Carrion's Disease in Monkeys.

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Mixed infections with *Bartonella bacilliformis* and plasmodia are not uncommon in regions where both malaria and Carrion's disease (verruga peruana, Oroya fever) are endemic. Both parasites induce swelling of the spleen, and both invade the red blood cells. It is conceivable that the preexistence of malaria might have an unfavorable influence upon a subsequent infection with *Bartonella bacilliformis*, as a result of impairment of the defensive powers of spleen and blood by the plasmodia, or that the introduction of plasmodia during the course of a dormant or partially controlled verruga infection might result in renewed invasion of the blood and lymphatic system by *Bartonella bacilliformis*. The abrupt development of malignant Oroya fever in the course of apparently benign verruga would be explicable on this latter hypothesis.

The opportunity to obtain definite experimental data on the effect of malaria infection on the course of experimental verruga presented itself when malaria parasites were found in the blood of a monkey (*Macacus cynomolgus*) which had been splenectomized in connection with an experiment to determine what influence the operation would have upon the susceptibility of monkeys to *Bartonella bacilliformis*.

Protocol 1.—Macacus cynomolgus 1-M, splenectomized on Dec. 1, 1926, to serve as control for *M. cynomolgos 2-S*, which was splenectomized the same day and inoculated with *Bartonella bacilliformis*. Examination of the blood of Monkey 1-M was made daily in order that the appearance of any *Bartonella*-like intracor-

puscular elements (such as appear in splenectomized rats[1]) might be detected. These elements were not found, but the examinations revealed plasmodia[2] on Dec. 6, 9, 10, 11, and 16, 1926, and the parasites were still present at the time of death on Mar. 10, 1927. No fever was detected in this animal at any time. The monkey had shown no signs of illness during several months of captivity previous to the operation. It would appear that it was a chronic malarial parasite carrier, a condition already established for man. Death may have been due to aggravation of the malarial infection through splenectomy.

Preexisting or Concomitant Malarial Infection and Verruga.

A *Macacus rhesus* intravenously inoculated with the blood of *Macacus cynomolgus 1-M* on December 10 soon developed fever, and the malarial parasites were found in the blood. One month later verruga material was intradermally inoculated. In due course the cutaneous lesions appeared and attained moderate size. Blood taken on February 3, 1927, yielded cultures of *Bartonella bacilliformis*, but no severe form of blood invasion and anemia developed. The animal recovered from both infections within a few weeks.

Protocol 2.—Macacus rhesus 2-M, inoculated intravenously with 0.5 cc. of the blood of Monkey 1-M on the day when plasmodia were first noticed (Dec. 10, 1926). The febrile reaction began on Dec. 15, that is, 5 days after inoculation, and continued daily for 9 successive days, being high (105–106.2°F.) for 5 days and moderate (104.2–104.6°) for 4 days longer. The plasmodia were present on Dec. 16. The temperature was normal (about 102°) from Dec. 26 to Jan. 3 but rose to 104.6° on Jan. 4, 1927, and to 106° on Jan. 5. Plasmodia were found in the blood on Jan. 3. After Jan. 5 there was no more fever. On Jan. 10, 1927, cultures of *Bartonella bacilliformis* and a saline suspension of the nodule of *M. rhesus 73* were intradermally inoculated. The signs of verruga were already evident on Jan. 19, and by Feb. 3 the nodules were of moderate size, and the blood had a culture titer of 1:100. The animal had no further paroxysm of malaria and recovered from both infections within a few weeks.

Another monkey was inoculated simultaneously with malaria and verruga material.

Protocol 3.—Macacus rhesus 3-M was inoculated on the same day as *M. rhesus 2-M* with 0.5 cc. of blood containing plasmodia and in addition received, both

[1] Mayer, M., *Arch. Schiffs.- u. Tropenhyg.*, 1921, xxv, 150. Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Woch.*, 1926, v, 559; *Arch. Schiffs.- u. Tropenhyg.*, 1927, xxxi, 295. Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 235.

[2] The parasite would appear to be *Plasmodium inui* (Wenyon, C. M., Protozoology, New York, 1926, ii, 968–973).

intravenously and intradermally, cultures of *Bartonella bacilliformis* and a suspension of verruga tissue. The animal's blood showed plasmodia on Dec. 16, 1926, 3 days before the onset of fever. The first paroxysm lasted 3 days (temperature 104.6°, 106.2°, 106.2°). The second rise of temperature (to 105.4°) was first detected on Dec. 27, temperatures not being taken daily during the Christmas holidays, and fever was still present on Dec. 28 (104°). Plasmodia were present in the blood on Jan. 3, and there was fever on Jan. 4, 5, and 6 (104°, 105.8°, 104°). Verruga nodules arose at the sites of intravenous inoculation into the saphenous veins (where several unsuccessful punctures had been made in the course of intravenous injection), but none at the sites of intradermal injections on the abdomen. Blood cultures made on Dec. 21, 1926, failed to yield cultures of *Bartonella bacilliformis*. The temperature of 106.2°F., recorded on that day, may have been due either to malaria or to verruga. A second injection of virulent verruga material intradermally on Jan. 10, 1927, resulted in only slight induration at the sites of inoculation, but the nodules on the back of the legs reached moderate size, and marked edema of the scrotum developed. The animal recovered.

The experiments outlined gave no evidence that the malarial infection in *Macacus rhesus* influenced unfavorably the result of infection with *Bartonella bacilliformis* when the plasmodia were introduced a month previous to inoculation with verruga or when both parasites were simultaneously inoculated.

Malarial Infection during Convalescence from Verruga.

The lesions resulting from experimental infection with *Bartonella bacilliformis* in monkeys usually heal within a few months, the microorganisms persisting longest in the spleen and lymph glands, where they can be demonstrated late in convalescence. It was of interest to determine whether, at this stage, the infection would again become active as a result of introduction of plasmodia.

As the protocols show, the inoculation of malaria parasites during convalescence had no influence whatever upon the course of recovery. In one instance the animal was reinoculated with virulent verruga material a month after the introduction of the plasmodia, but reinfection was not induced. The malarial infection apparently had not interfered with the development of immunity.

Protocol 4.—Macacus rhesus 4-M had had an infection with *Bartonella bacilliformis* during the autumn, but the skin lesions had almost healed. It was inoculated with 0.5 cc. of the malarial blood intravenously on Dec. 10, 1926. There

was fever lasting for 6 days, Dec. 22 to 27, 1926 (temperatures varying from 104.4° to 105°), and the blood was positive for plasmodia on Jan. 3, 1927. Blood culture was negative for *Bartonella bacilliformis* on Dec. 21, 1926, and the skin lesions had disappeared.

Macacus rhesus 5-M, also recovering from verruga, was inoculated with 0.5 cc. of malarial blood at the same time as the foregoing animal. Fever began on Dec. 16, 1926, and lasted 5 days (temperatures 104°–106.8°). Plasmodia were present on Dec. 16, 1926, and on Jan. 3, 1927. On Dec. 28, 1926, the temperature rose to 104.4° but fell to 103.6° the next day, and there were no later paroxysms. On Jan. 8, 1927, verruga material was inoculated intradermally. No skin lesions appeared at the old sites, and none developed at the sites of the new inoculations, and the blood remained negative for *Bartonella bacilliformis* by culture. Observation ended Jan. 29, 1927.

Effect of Splenectomy upon Verruga Peruana.

In connection with the problem of the nature of the intracorpuscular bodies known as *Bartonella muris*,[1] which appear in the red cells of rats following splenectomy, it became of interest to determine what effect splenectomy would have upon the course of experimental infection with *Bartonella bacilliformis*.

A monkey splenectomized on November 12, 1926, and inoculated soon afterwards with verruga material, developed the severe type of Carrion's disease (Oroya fever), with marked anemia, extensive edema, emaciation, and skin lesions. Even more violent reactions than the one in this animal (*Macacus rhesus 1-S*) are occasionally observed in *rhesus* monkeys as a result of inoculation with *Bartonella bacilliformis*,[3,4] and hence the severity of the infection in this instance cannot be ascribed to the effect of splenectomy. Moreover, *M. cynomolgus 2-S*, which was subjected to splenectomy and simultaneously inoculated with nodular material from Monkey 1-S, showed a moderate reaction only. Although the blood titer was high (1:100,000), there were no other evidences of systemic infection, and the nodules were of medium size. The control monkey in this instance (*M. cynomolgus 5-T*) reacted more severely than the splenectomized animal, although the blood titer was only 1:10, as was that of a *rhesus* control.

[3] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.
[4] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

Protocol 5.—Macacus rhesus 1-S, splenectomized Nov. 12, 1926, was inoculated 3 days later intravenously with 1 cc. of culture of *Bartonella bacilliformis* and 1 cc. of saline suspension of nodular tissue from *Macacus rhesus 2-T*.[5] The same material was injected intradermally. A temperature of 104° to 104.6°F. was recorded on Nov. 11, 24, Dec. 10, 20, and 21, and on Jan. 15, 1927. Except on these 6 days the animal was afebrile. Blood cultures were positive on Nov. 24 (1:10,000), Dec. 8 (1:100,000), and Dec. 28 (1:100). The organisms were sufficiently numerous in the blood on Dec. 3 to be detected by examination of stained films. The nodules developed to moderately large size and persisted for about 2 months. From Dec. 11, 1926, to Jan. 3, 1927, there was general edema, especially marked in the scrotum, and a generalized nodular eruption.

Blood Counts.

	R.B.C.	Hemoglobin (Sahli) per cent
Nov. 12	5,456,000	90
Nov. 24	4,424,000	80
Dec. 27	3,080,000	50

The animal had completely recovered by Feb. 7, 1927.

M. cynomolgus 2-S, splenectomized Dec. 1, 1926, and inoculated intradermally with a culture of *Bartonella bacilliformis* and a saline suspension of nodular tissue from *M. rhesus 1-S*. Nodules of moderate size had developed by Jan. 14, 1927. Blood taken on Jan. 6, 1927, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules had disappeared by Feb. 9, 1927.

M. cynomolgus 5-T[5] was inoculated in the same way and with the same material as the foregoing animal but was not splenectomized. There was no fever at any time, but enormous cherry-red nodules developed on the eyebrows and abdominal wall, reaching their maximum growth on Dec. 27, 1926. The blood titer was 1:10 on Jan. 6, 1927, but blood culture was negative on Jan. 24.

Splenectomy did not induce relapse of experimental verruga infection in two animals which had recovered (*M. rhesus 40* and *41*[5]), nor did removal of the spleen render recovered animals susceptible to reinoculation with *Bartonella bacilliformis* (*M. rhesus 49, 50, 68*). Observation continued for a period of 1 month after inoculation of *Bartonella bacilliformis* into these animals failed to reveal any skin lesions, and blood cultures were uniformly negative throughout this period.

Effect of Splenectomy and Malarial Infection Together upon Verruga Peruana.

Two animals were subjected to splenectomy as well as to infection with plasmodia and *Bartonella bacilliformis*. One succumbed to the

[5] Noguchi, H., *J. Exp. Med.*, in press.

malarial infection before the verruga lesions had had time to develop; the other had a moderately severe infection with *Bartonella bacilliformis* and while recovering died of tuberculosis.

Protocol 6.—Macacus rhesus 6-M was splenectomized and at the same time inoculated with 0.5 cc. of malarial blood on Dec. 10, 1926. The plasmodia were found in the blood on Dec. 16, and 4 days later the temperature rose and remained high for 3 successive days (104.2–106°). The second paroxysm began on Dec. 29, 1926, and lasted for 8 days (temperatures 104–106.6°). The plasmodia were very numerous on Jan. 3. The animal was inoculated with verruga material on Jan. 8, 1927, but succumbed to the malarial infection on Jan. 14, 1927, before the verruga lesions had had time to develop. *Bartonella bacilliformis* was not recovered in blood cultures.

Macacus rhesus 7-M was splenectomized on Dec. 10, 1926, and at the same time inoculated with 0.5 cc. of malarial blood and 0.5 cc. of verruga cultures and tissue, the latter intradermally as well as intravenously. The first paroxysm occurred on Dec. 20 and lasted 4 days (temperatures 104.2–106°), the second, beginning Jan. 3, lasted 5 days (temperatures 104.6–106.4°). Examinations for plasmodia were positive on Dec. 16, 1926 (+), and on Jan. 3, 1927 (+++). Blood taken on Dec. 21, 1926, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000. The animal died of tuberculosis on Jan. 10, 1927.

SUMMARY.

The experiments reported were designed to determine the influence of malarial infection (*Plasmodium inui?*), splenectomy, or both combined, upon the course and character of experimental infection with *Bartonella bacilliformis* in monkeys (*Macacus rhesus* and *M. cynomolgus*).

Blood withdrawn from a monkey showing spontaneous malarial infection was inoculated intravenously into monkeys (*a*) 1 month prior to inoculation with virulent verruga material, (*b*) simultaneously with the verruga material, and (*c*) during convalescence from verruga infection of moderate severity. All the monkeys contracted the malarial infection and suffered one to three paroxysms during a period of about a month. The verruga lesions appeared in the inoculated animals in due course, were of average size, remained for the usual length of time, and *Bartonella bacilliformis* was recovered in culture from blood which also contained the plasmodia. The lesions in the convalescent animals continued to heal at the normal rate, and blood cultures were negative for *Bartonella bacilliformis*, as is usual during

convalescence. One of the recovering animals was reinoculated with virulent verruga material a month after the injection of the malarial blood, but neither did new lesions arise nor old ones recur. The malarial infection, therefore, had no effect upon the course of verruga or upon the establishment of immunity to *Bartonella bacilliformis*, hence it would appear that malaria and verruga may coexist in the same individual without unfavorable effect of one disease upon the course of the other.

Similarly, splenectomy led to no appreciable aggravation of *Bartonella* infection. One monkey subjected to splenectomy and inoculated with verruga material shortly afterwards had an unusually severe reaction, but another, which was infected with material from the first and simultaneously splenectomized, reacted only moderately, while the non-splenectomized control showed a severer type of cutaneous infection. Even the combination of splenectomy and malarial infection did not appreciably aggravate the experimental verruga. Neither relapse of verruga nor reinfection with *Bartonella bacilliformis* was induced in convalescent or recovered monkeys as a result of splenectomy.